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THE DEGRADATION OF ASCORBIC ACID IN
SASKATOON BERRIES (AMELANCHIER ALNIFOLIUS)

by



MARGUERITE PANTHER

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled THE DEGRADATION OF ASCORBIC ACID IN SASKATOON BERRIES (Amelanchier alnifolius) submitted by Marguerite Panther in partial fulfilment of the requirements for the degree of Master of Science.

DATE

June 9, 1971

ABSTRACT

An examination of the fruit of the saskatoon, Amelanchier alnifolius (using both colourimetric and polarographic assay procedures) showed L-ascorbic acid (vitamin C) to be absent. However, the reduced form of the compound, dehydroascorbic acid, was detected.

When synthetic ascorbic acid was added to extracts from the saskatoon berries, polarographic assays showed rapid degradation. This phenomenon was found to be greatest in the immature fruit and gradually decreased with the onset of maturity for all varieties of saskatoons examined. The factors involved in this degradation were sensitive to higher temperatures but were not affected by freezing at -12°C .

Investigations into the causes which could explain this degradation of ascorbic acid, revealed that an ascorbic acid oxidase enzyme was present, which catalysed the conversion of ascorbic acid to the dehydro form. Subsequent work was directed into the non-enzymic causes for the degradation of the antiscorbutic factor.

The effect of added copper ions on the saskatoon system was complicated because ascorbic acid oxidase has a copper containing prosthetic group, and the metal affects both the stability of ascorbic acid and the plant pigments. Two separate systems were found to exist in this context, depending on the pH of the extraction medium.

Protection of ascorbic acid degradation was achieved when anthocyanidins, flavonols and other phenolic components were added to saskatoon extracts, the degree of protection depending on the variety of fruit

and also the compound added.

From the data collected the complexity of the degradation systems is evident. Two systems were found to exist, but because of the large number of inter-related factors the systems were not analysed individually.

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First get your facts,
and then you can
distort them at
your leisure

Mark Twain

I. INTRODUCTION

In addition to major nutrients such as carbohydrates, proteins, fats, minerals and water, every animal being requires for its well being and normal growth some important accessory food factors (Hopkins 1912). Originally these compounds were believed to be amines and so the denotation "vitamins" has been reserved for these food factors.

Most vitamins are directly or indirectly supplied to the animal body by plants which are capable of producing them. One of the active components in fresh vegetables is ascorbic acid. This history of this accessory food factor dates back to the 15th century, without anyone being aware of the existence of a specific compound until the early 1900's.

Deficiency or absence of this compound results in a disease called scurvy. The amount of ascorbic acid required for alleviation of the scorbutic symptoms is a subject of controversy, as are the physiological effects of ascorbate deficiency. Dr. Linus Pauling (1970) has challenged the fields of nutrition and medicine by advocating an intake of 200 - 1000 mg of ascorbic acid per day to decrease the incidence and severity of the common cold. Controversy continues over the actual amount required, but the fact that ascorbic acid is needed by man is well established.

The study described below was an investigation into the relationship of ascorbic acid and saskatoon berries (Amelanchier alnifolius). This species of shrub is widespread in North America and grows over a

vast range of latitude. Only recently has the plant been cultivated in Alberta on a commercial scale for the production of wine, jams, jelly and piefillers from the fruit.

The ascorbic acid content of the berries was found to be negligible. However when berry extracts were fortified with ascorbic acid, degradation of the added vitamin was observed. Further work was directed in an attempt to solve this complex oxidative process, in the light of information which exists to date, in terms of enzymic and non-enzymic changes taking place in the saskatoons.

II. REVIEW OF LITERATURE

A. Ascorbic Acid and Man

(1) Ascorbic acid as an accessory food factor

Ascorbic acid or vitamin C has a more limited distribution than other water soluble vitamins. Citrus fruits, currants, berries and green vegetables are the richest sources with traces found in a variety of other foods. (Table 1)

Attempts were made during the 1930's - 1940's to augment regular sources of vitamin C with unusual, naturally occurring materials. Tuba, Hunter and Kennedy (1944) used rosehips for this purpose. They found that quantities of ascorbic acid present in rosehips depended on the species of plant and also the place of cultivation (Table 1). In the U.S.S.R., Krotkov (1943) investigated other naturally occurring sources of the vitamin, such as pine-needles and green walnuts.

A survey among students at the University of Alberta (Waagen and Pett, 1942) and a dietary survey in Edmonton (Hunter and Pett, 1941) showed a definite deficiency of ascorbic acid in the diet, except from June to September. However, with the increased use of transportation, fresh fruit and vegetables from the United States of America and Mexico are available throughout the year, so it is unlikely that the deficiency exists today.

Root vegetables and potatoes contain small amounts of ascorbic acid, but because of the amount normally consumed everyday the latter represents an important source of the vitamin. This is particularly

TABLE 1

The ascorbic acid content of some food materials

Food Material	Vitamin C in mg/100 gm fresh weight	Reference
Chokecherry	5	Tuba, Hunter and Kennedy 1944
Rosehip: <i>Rosa acicularis</i>	1786	Tuba, Hunter, Hutchinson and Kennedy, 1944.
<i>Rosa woodsii</i>	1317	ibid.
<i>Rosa arkansana</i>	1317	ibid.
<i>Rosa blanda</i>	724	ibid.
Black currants	250	Kuusi, 1965
Orange - flavedo	230	Rauen, Devescovi and Magnani, 1943
albedo	140	ibid.
fruit flesh	60	ibid.
Apple	10	Johnson, Spangelo, Watkins and Hammill, 1968
Strawberry	60	U.S. Department of Agriculture 1950
Cabbage	60	ibid., 1964
Milk (per litre)	15-25	Mihelic and Mikic, 1961
Beef liver	31	Schweigert and Payne, 1956
Pork liver	23	ibid.
Beef lung	0	ibid.
Seal adrenal	127	Hoygaard and Rasmussen, 1939
Muscle meat	2	McCance and Widdowson, 1960

the case in Britain and other European countries where the potato is a staple food. Table 2, after Sweeney, Hepner and Libeck (1969) gives the ascorbic acid content of different varieties of potatoes as affected by storage time and temperature.

Meat, eggs, milk, and other foods of animal origin contain only small amounts of ascorbic acid. Hoygaard and Rasmussen's (1939) analysis of the vitamin C content of various seal tissues revealed that the richest source of vitamin C in these animals are the adrenal glands. (Table 1) Sir Robert McCarrison (1919) noticed that these glands are hypertrophied in scorbutic guinea pigs and suggested high in vitro concentrations of the vitamin inhibit the enzymes responsible for the hydroxylation reactions essential to the formation of cortical hormones. Injected adreno-corticotrophic hormone leads to a rapid fall in ascorbic acid in the adrenal glands, and this is followed by the secretion of the cortical hormones (Meiklejohn, 1953).

The roe of fishes and other glandular fishy tissues give protection to such people as the Eskimos, whose diet consists of little else than meat and fish. Beef liver has been found to contain 30 mg ascorbic acid/100 gm of raw flesh, and so it is an important source of the vitamin (McCance and Widdowson, 1960).

Most animals can synthesise ascorbic acid themselves; according to Chatterjee, Kar, Ghosh and Guha (1961) the synthesis is probably via D-glucose and the D-glucuronic acid pathway. During evolutionary ascent, it seems that the enzymes responsible for the synthesis of ascorbic acid, originally resided in the kidney. The liver gradually becomes the organ responsible for the synthesis of the

TABLE 2

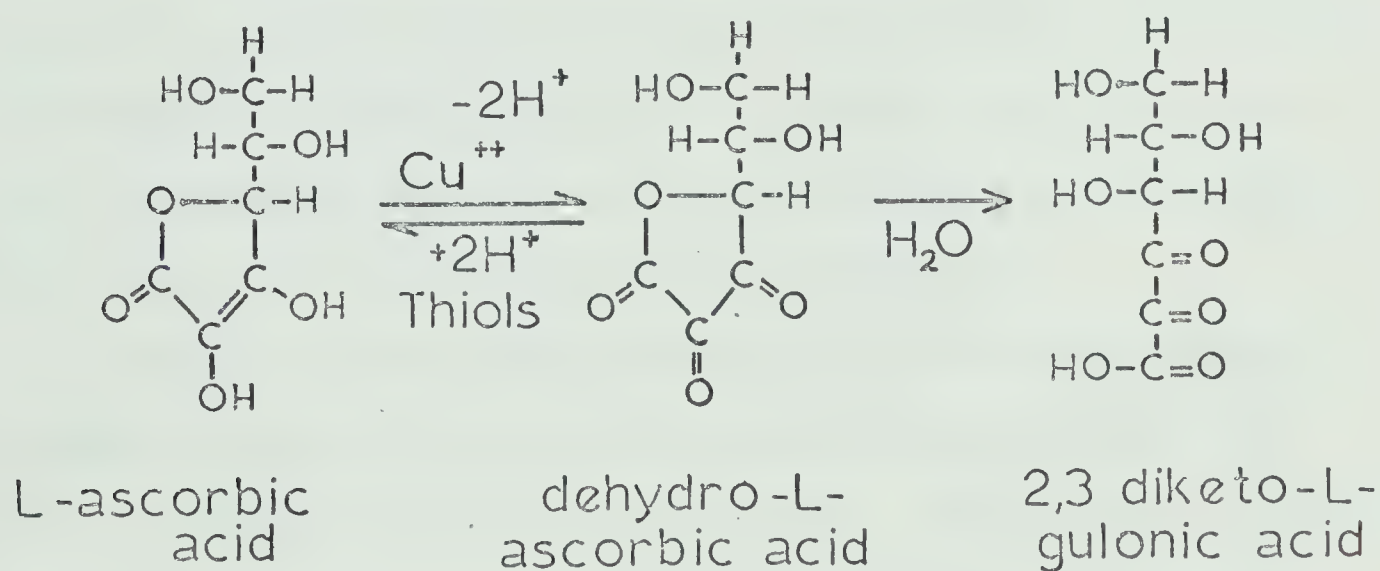
The ascorbic acid content of different varieties of potatoes
as affected by storage time and temperature
(Sweeney, Hepner and Libeck, 1969.)

Year	Section	Storage Time (Months)	Storage Temperature (°F)	Ascorbic Acid mg/100 gm		
				Pungo	Rose- mont Cobbler	Russet Burbank
1965	Stem	0		22.5	21.2	18.3
		1	70	16.5	15.5	14.8
		2	55	13.6	13.4	13.7
		5	55	8.5	9.2	10.8
	Bud	0		25.6	25.5	22.9
		1	70	17.9	17.6	18.0
		2	55	14.2	15.4	16.2
		5	55	10.0	12.0	11.5
				Pungo	Irish Cobbler	
1966	Stem	0		34.1	24.8	
		1	70	24.8	18.4	
		2	55	21.0	13.1	
		5	55	12.6	10.8	
	Bud	0		47.6	29.4	
		1	70	31.2	22.6	
		2	55	24.3	16.4	
		5	55	14.1	13.2	

vitamin, and finally the synthetic ability disappears from the liver also. (Chatterjee, Chatterjee, Ghosh, Ghosh and Guha, 1961) Man is one of the higher primates who is unable to carry out the synthesis of L-ascorbic acid.

An intake which is considered adequate varies from country to country. In the United States of America, the recommended dietary allowance of ascorbic acid is 55 mg per day for women and 60 mg per day for men (Goldsmith, 1968), while in Canada, the requirement is 30 mg per person per day.

The structure of ascorbic acid and related compounds of less antiscorbutic value is given below.



(2) Some of the effects of a deficiency of the vitamin

The characteristics of scurvy as a result of ascorbic acid deficiency, are loosening of the teeth through lack of cement that binds them, swelling of the joints and various types of haemorrhages. These features can be attributed to defects in collagen synthesis. Collagen represents the main supportive protein of the skin, tendons,

bone cartilage and connective tissue. Ascorbic acid is said to control the hydroxylation of amino acids, particularly proline after they have been incorporated into the polypeptide chain of the collagen fibrils (Stone and Meister, 1962).

Coronary atherosclerosis is a distinct pathological condition associated with the coronary and cerebral arteries and also the aorta. The histology is characterized by lesions with a high cholesterol content localized in the subendothelial stratum of the arterial intima (Duguid, 1949). When deficiencies of ascorbic acid are induced, the pathological conditions of atherosclerosis are reproduced because the primary alterations in atherosclerosis are morphologically of the intimal ground substance (Willis, 1953). Work in this context by Ginter, Bobek, Babala and Barbierikova (1969) indicated an accumulation of cholesterol in guinea pigs which were hyposaturated with ascorbic acid. This finding is presumed to be connected with a certain role played by ascorbic acid in cholesterol catabolism.

Elliot and Smith (1966) found that the presence of fluoride decreased the loss of ascorbic acid from guinea pigs leucocyte cells and also stimulated the uptake of the vitamin. Boruch, Jervis, Elliot, and Smith (1968) speculated that this effect of ascorbic acid was related to the permeability of cell membranes. Cells having a higher concentration of ascorbic acid, permitting much freer diffusion of fluoride.

Anaemia is a common, but inconstant feature of scurvy. Since patients with scurvy are often anaemic, Bronte-Stewart (1953) suggested that ascorbic acid is concerned with erythropoiesis. Goldberg (1961)

has shown that if isotopically labelled red blood cells from a healthy person are injected into patients with scurvy, they have greatly diminished life. Red cells from scorbutic patients if injected into healthy persons survive normally. These results suggest that a haemolytic state develops in scurvy and is responsible for the anaemia.

B. Ascorbic Acid and Higher Plants

(1) The biosynthesis of L-ascorbic acid in plants

The first convincing evidence that ascorbic acid is formed from a hexose was provided by the experiments of Ray (1934) with excised pea-embryos growing on a synthetic medium, hexose sugars increased the production of this vitamin. The hexoses are not only convertible but give rise to a variety of metabolites among which the substances acting as precursors might be found.

When D-glucose, labelled on C-1 was injected into detached ripening strawberry fruit, Loewus, Finkle, and Jang (1958) found the six carbon chain of the sugar was preserved and no inversion had taken place. The scheme proposed by these workers for the biosynthesis of ascorbic acid is given in Figure 1.

(2) Present concepts of the function of ascorbic acid

There is a paucity of information regarding the function of ascorbic acid in vivo. Satô and Kasai (1969) exposed young grape berries to radioactively labelled carbon dioxide for ten minutes. Light and dark conditions were used and the changes in the labelled assimilation products was surveyed. Tartaric acid was formed from sugars derived from the labelled CO₂ fixation products, and the C₁ - C₄

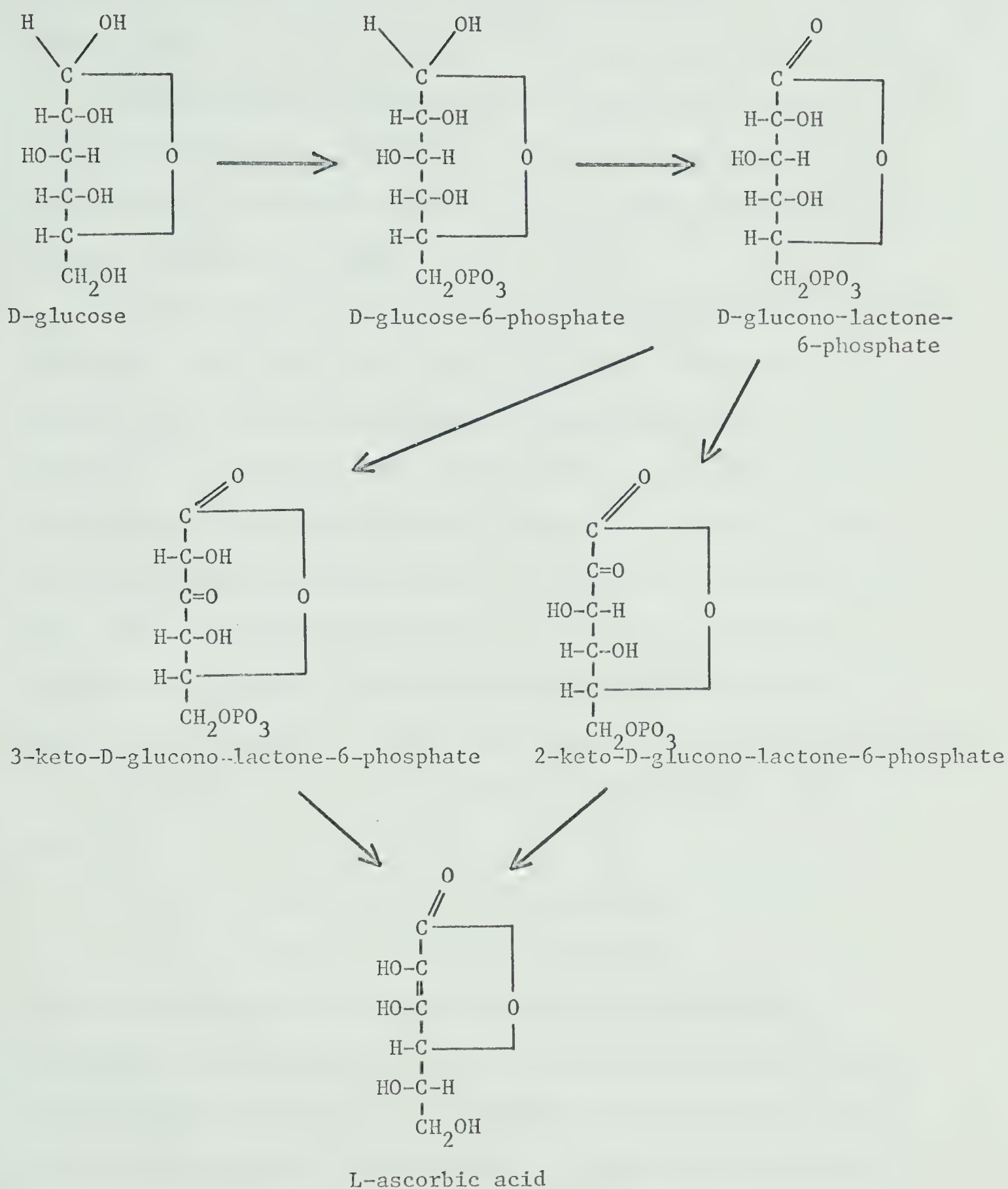


Figure 1. The biosynthesis of L-ascorbic acid in plants.
(Loewus, Finkle and Jang, 1958)

moiety of L-ascorbic acid was identified as one of the sources of tartaric acid.

In the oxidation of such monohydric phenols as p-coumaric acid to caffeic acid, Kubowitz (1938) found that an induction period is present before the reaction starts. Kreuger (1958) used ascorbic acid to remove or shorten the phase.

In plants there is a close association between glutathione (GSSG) and ascorbic acid (AA). Hopkins and Morgan (1943) found these two compounds to appear at the same time in both germinating seeds and sprouting tubers, and plant tissues were found to contain the enzyme dehydroascorbic acid reductase which catalyses the transfer of the proton from reduced glutathione (GSH) to dehydroascorbic acid (Crook, 1941). This scheme has been linked with oxidative enzymes which catalyze the conversion of ascorbic acid (AA) to dehydroascorbate (DHA), indicating the possibility of hydrogen transfer from GSH to oxygen via the ascorbic-dehydroascorbic acid system, according to the following scheme:



Direct evidence that there is a connection between the state of oxidation of reduced glutathione and that of ascorbic acid was shown by Barker and Mapson (1951), in experiments involving potato tubers stored in an atmosphere of pure oxygen. A fall in the glutathione content was found followed closely by a fall in the ascorbic acid content and a rise in dehydroascorbic acid. These workers suggested that the ascorbic acid content of the tissues is dependent on the maintenance of its reduced glutathione content. Mapson and Goddard

(1951) found reduced coenzyme II and glutathione reductase to be involved in the scheme also. The reaction was as follows:



Chinoy (1967) presented evidence for the involvement of ascorbic acid with nucleic acids and protein metabolism, in a general concept of floral induction and differentiation. Experiments showed that under inductive photoperiod and temperature there was a rapid upsurge in production and utilization of ascorbic acid in the shoot apex at the time of floral initiation as well as during the period of reproductive differentiation.

According to this concept extended by Chinoy and Mansuri in 1966, the metabolism of ascorbic acid at enhanced levels produces a highly activated metabolic state in the shoot apex at the time of floral induction as well as subsequently into the developing spike during the period of reproductive differentiation. Consequently deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and hence proteins and enzymes, are synthesised at faster rates, resulting in the quicker laying down of growth centres, as well as increased rates of cell division and cell enlargement. At a certain critical stage, synthesis of nuclear constituents gains a precedence over that of cytoplasmic and cell wall materials in some cells, and thus a change in the norm of mitotic division is brought about leading to meiosis.

Chinoy (1967) considered the most noteworthy finding relating to ascorbic acid metabolism of the shoot apex is the pronounced ability of ascorbic acid to form complexes with DNA, proteins and perhaps other organic molecules of moderate size. Such complexing of ascorbic acid

with DNA has been achieved in vitro.

Chinoy (1967) visualizes the mechanism underlying the complex phenomenon of floral induction and differentiation to involve the monovalent oxidation of ascorbic acid in aerobic conditions, with the aid of a special peroxidase and production of a powerful reducing agent, monodehydroascorbic acid. This is a free radical and might serve as an electron donor in the biosynthesis of macromolecules. Photophosphorylation as well as oxidative phosphorylation are also energized by the flow of electrons from monodehydroascorbic acid thus augmenting the pool of high energy phosphate. Dehydroascorbic acid formed from the loss of an electron from monodehydroascorbic acid is again reduced to ascorbic acid with the help of a reductase.

Chinoy (1967) postulated that the nucleoprotein complexes with ascorbic acid to form a charge transfer complex. The rate of complex formation increases considerably during the period of reproductive differentiation, thus increasing the direct flow of electron energy for metabolic processes, leading to intense cellular activity, culminating in flowering. Fejer, Johnston, Spangelo and Hamill (1970) studied the ascorbic acid content of both raspberry fruit and leaves. The content was found to be higher in the latter although in both the fruit and leaves the ascorbic acid content showed seasonal variation. There seemed to be no possibility to select for leaves with a high ascorbic acid content, hoping this indicated the vitamin content of the fruit. There were indications, however, that the content of the leaf is inherited by the seedlings. Fejer et al. (1970) related a high ascorbic acid content in the leaves to early leaf fall. These workers

explained that the ascorbic acid content of the fruit is correlated with the earliness of leaf fall by the hormone status of the plants and is related to the hypothesis on flowering of Chinoy (1967) mentioned earlier.

(3) The degradation of ascorbic acid

a. Enzymic degradation in plants and microbes

There are many enzymes in plant tissues which possess an oxidising function and it is quite conceivable that many play some role in deteriorative processes, albeit a definite link has been established in only a few cases.

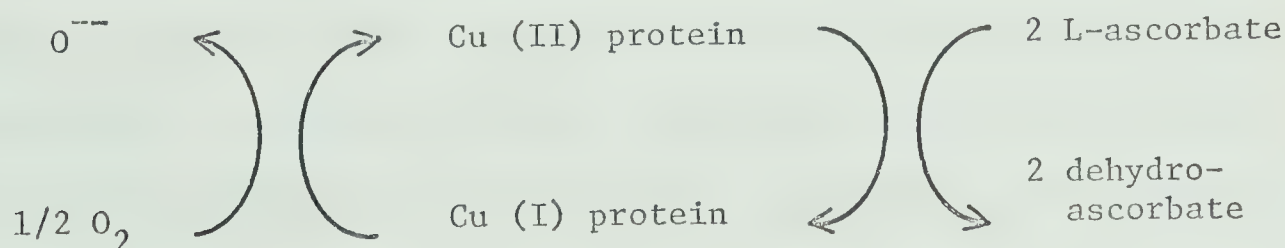
A very active ascorbic acid oxidase which converts ascorbic acid to dehydroascorbic acid (Page 7) has been isolated from the yellow crook-neck squash Cucurbita pepo. Tokuyama, Clark, and Dawson (1965) found the enzyme to have a molecular weight of 140,000 between pH 5.7 - 8.7. The natural substrate of the enzyme is assumed to be ascorbate, but it is also active towards ring analogues with a dienol grouping adjacent to a carbonyl group and substituted with polyhydric and amino phenols.

The enzyme has been detected in the soluble fraction from plant tissues (Bonner, 1957) although Vines and Oberbacher (1963) found the enzyme in the cell debris. Butt and Hallaway (1960) found the distribution between the two fractions varies with the type of tissue; in general, more is associated with the cell wall in preparations from mature tissues than from young tissues. However, the in vivo enzyme may be wholly associated with one site since during extraction some of the activity could be lost from that site and appear in a second subcellular fraction. Hallaway, Phethean, and Taggart

(1970) concluded that the ascorbic acid oxidase activity of the cell wall and soluble fraction prepared from leaves of cabbage (Brassica oleracea) is attributed to the same enzyme and that in vivo the majority of the enzyme is located in the cell walls.

Ascorbic acid oxidase (L-ascorbate: O_2 oxidoreductase) is a conjugated copper protein which mediates the direct transfer of electrons from its substrate L-ascorbic acid to molecular oxygen. While it has been shown that the prosthetic copper is essential to the oxidase activity, the precise role of copper is yet to be elucidated. Although in the enzymic reaction several copper ions on the enzyme are reversibly oxidized and reduced during the catalysis. An oxidized form of the enzyme reacts with ascorbic acid to give the ascorbate free radical. Yamazaki and Piette (1961) detected this radical by means of electron spin resonance spectroscopy. In a later step the radical is oxidized further to dehydroascorbic acid. The oxidized form of the enzyme is then regenerated by reaction with oxygen.

A number of properties of prosthetic copper have been defined and Magee and Dawson (1962) have proposed that a reversible copper valency change occurs during enzyme function, i.e.



It would seem that the functioning state involves a configurational change in the enzyme such that the copper prosthetic group becomes more reactive. Joselow and Dawson (1951) have shown that the prosthetic

copper is available for exchange with ionic and radioactive copper only while the enzyme is functioning. The inactivating effect of hydrogen peroxide is most pronounced when the enzyme is in the functioning state (Tokuyama and Dawson, 1962). However, Poillon and Dawson (1963) conclude that the hydrogen peroxide inactivation was attributable to a 25% fraction of the prosthetic copper that existed in the Cu (I) state. These investigators also used reagents specific for either the Cu (I) or Cu (II) valency state and they concluded the prosthetic copper is of mixed valency--25% Cu (I) and 75% Cu (II). When they assayed the enzyme in the presence of bathocuproine, which specifically complexed the 25% Cu (I) fraction, they demonstrated that this fraction was not involved in the oxidation of L-ascorbic acid.

Clark, Poillon and Dawson (1966a) showed at pH of 3.6 a loss of both ascorbic acid oxidase activity and of its copper content. This occurred in a non-parallel fashion. The activity is lost faster than the copper ions which suggested that the enzymatic Cu (II) fraction may be lost at a faster rate than the non-enzymatic Cu (I). However, the non-parallel loss in activity and copper content is consistent with the view that the enzyme is only fully active when the prosthetic copper atoms exist and function in specially oriented groups of two or more. Consequently, the loss of a single copper atom would cause loss in activity proportionately higher than the copper loss. The changes found by Clark, Poillon and Dawson (1966a) were temperature dependent and were accompanied by an irreversible unfolding, with subsequent aggregation of the protein moiety of the enzyme. Conversely, at pH 10.4 or higher an apparent dissociation of the enzyme occurred without

change of copper content, although the characteristic absorbance at 604 mμ was lost. Molecular weight data indicated the existence of at least two sub-units in the enzyme.

Later ultracentrifugal studies by Clark, Poillon and Dawson (1966b) have shown that native reduced or hydrogen peroxide inactivated ascorbate oxidase have essentially identical sedimentation coefficients, $S_{20,w} = 5.7 - 5.8$. The apoenzyme was also found to have similar sedimentation coefficients. The work of Clark et al. (1966b) indicated that gross structural changes had not occurred under the different conditions used in these experiments, so it was concluded that the prosthetic copper probably does not have a direct role in maintaining the conformation of ascorbic acid oxidase.

Porath, Samorodova-Bianki and Hjerten (1967) believe the above work which generally involved the method of Dawson and Magee (1955) was not of sufficient accuracy, since the preparation of ascorbate oxidase used was of inadequate purity. A purer preparation of ascorbate oxidase was obtained by these workers, using the method of column fractionation on Sephadex dextran gel (Porath, 1962) and agarose (Hjerten, 1962). Cucumber extract was found to contain at least three markedly different forms of ascorbate oxidase, two of high molecular weight (200,000 - 900,000 if it is assumed that they are of globular form) and one with a low molecular weight of about 10,000. The possibility must not be ignored that the three fractions may be stable polymers of one and the same low molecular weight protein. However, they may have a more complex structure. Amon and Markakis (1969) did separate the ascorbate oxidase of the yellow summer squash into five isozymes by polyacrylamide gel

electrophoresis. One of the forms accounted for 70 per cent of the recovered total enzymic activity.

The effect of flavonols such as quercetin, dihydroquercetin, morin and rutin were found by Porath, Samorodova-Bianki and Hjertin (1967) to inhibit all forms of the enzyme, whereas, previous work by Stenlid and Samorodova-Bianki (1966) showed that the activity of the third fraction was increased. The material most often used in studies of ascorbic acid oxidase is the squash plant, Cucurbita pepo. However, enzyme systems capable of oxidizing L-ascorbic acid are found in many other plants. The copper protein, "ceruloplasmin", of human serum (Osaki, McDermott and Frieden, 1964) represents an animal source. White and Krupka (1965) report two ascorbic acid oxidizing enzyme systems present in the mycelium of the fungus of Myrothecium verrucaria. One of these enzymes was studied previously by White and Smith (1962) and was termed "atypical" because it was unaffected by inhibitors of heavy metal catalysis in contrast to the copper containing enzyme of higher plants. One of the systems which White and Krupka (1965) found in this fungus, for which the name ascorbic acid oxygenase is suggested, catalyses the reaction:



A similar system had not been reported before. The enzyme appears to be specific for L-ascorbic acid as opposed to the close analogue D-isoascorbic acid. Ferrous ions which are weakly bound to the protein are required for activity. Nevertheless, cyanide and ethylenediamine tetraacetate are not inhibitory. The second ascorbate enzyme present in the fungus is also strange in that it is not markedly inhibited by

cyanide. It catalyses the following reaction:



The enzyme oxidises ascorbic acid and several of its close analogues (e.g., L-lyxonic and xylonic acids) and requires oxygen as an electron acceptor. It is not inhibited by metal chelators such as diethyldithiocarbamate, sulphhydryl reagents or flavin enzyme inactivators and does not lose activity or dialysis against cyanide or ethylenediamine tetroacetate (EDTA). In the presence of ascorbic acid and oxygen, the oxidase is rapidly inactivated by high concentrations of hydrogen peroxide ($5.6 \times 10^{-3}\text{M}$) or by Fenton's reagent ($\text{Fe}^{++} + \text{H}_2\text{O}_2$). The enzyme activity is progressively lost during the oxidation of ascorbic acid. This loss, termed "reaction inactivation" is not due to inhibition by reaction products or to the formation of free hydrogen peroxide. Hydrogen peroxide at low concentrations ($5.6 \times 10^{-4}\text{M}$) stimulates oxidase activity.

Volk and Larsen (1963) discovered the presence of a bacterial ascorbate oxidase when a strain of Aerobacter aerogenes was grown with either glucose or ascorbate as a carbon source, whether under aerobic or anaerobic conditions. Organisms such as Aerobacter aerogenes, Escherichia coli, Bacillus megaterium, Micrococcus lysodeicticus, Propionibacterium pentosaceum and Gaffkya tetragena taken from the departmental culture collection were unable to oxidise ascorbic acid. The bacterial enzyme described by Volk and Larsen (1963) is a specific ascorbate oxidase which appears to have a limited substrate specificity. The insensitivity to carbon monoxide as well as the low oxygen affinity serve to differentiate this enzyme from cytochrome oxidase and phenol oxidase.

b. The non-enzymic degradation of ascorbic acid

Since the catalytic activity of ascorbic acid oxidase depends upon a copper containing prosthetic group, the possibility cannot be ignored of non-specific metal catalysis of various reactions before or after inactivation of the enzyme. Despite this, it has been known for some time that ascorbic acid is oxidized by oxygen even in the absence of the enzyme, but is a comparatively slow reaction. However, the rate of the non-enzymic reaction is increased markedly if transition metal ions especially copper (Cu II) or iron (Fe III), are present in catalytic amounts.

Kahn and Martell (1967a) have obtained good kinetic evidence that an intermediate in the metal ion catalyzed reaction is a complex of ascorbate monoanion, oxygen and the metal ion similar to B in Figure 2. These workers suggested that the rate determining step for the reaction is the transfer of an electron from the complexed ascorbate to the complexed oxygen to give a species such as C in Figure 2. This compound would be a resonance hybrid of B (Figure 2) because it merely represents the product of electron shifts. The reaction would not be completed until some atom moves. However, Hamilton (1969) suggests a more likely mechanism, that is, the transfer of electrons and a proton to give a compound similar to D in Figure 2. This could then be expected, as illustrated to give Cu (II), dehydroascorbic acid and hydrogen peroxide rapidly.

From studies using several chelating agents, Kahn and Martell (1967b) found that the oxidation of ascorbic acid does not appear to occur by the same mechanism. Examples of chelating agents used are

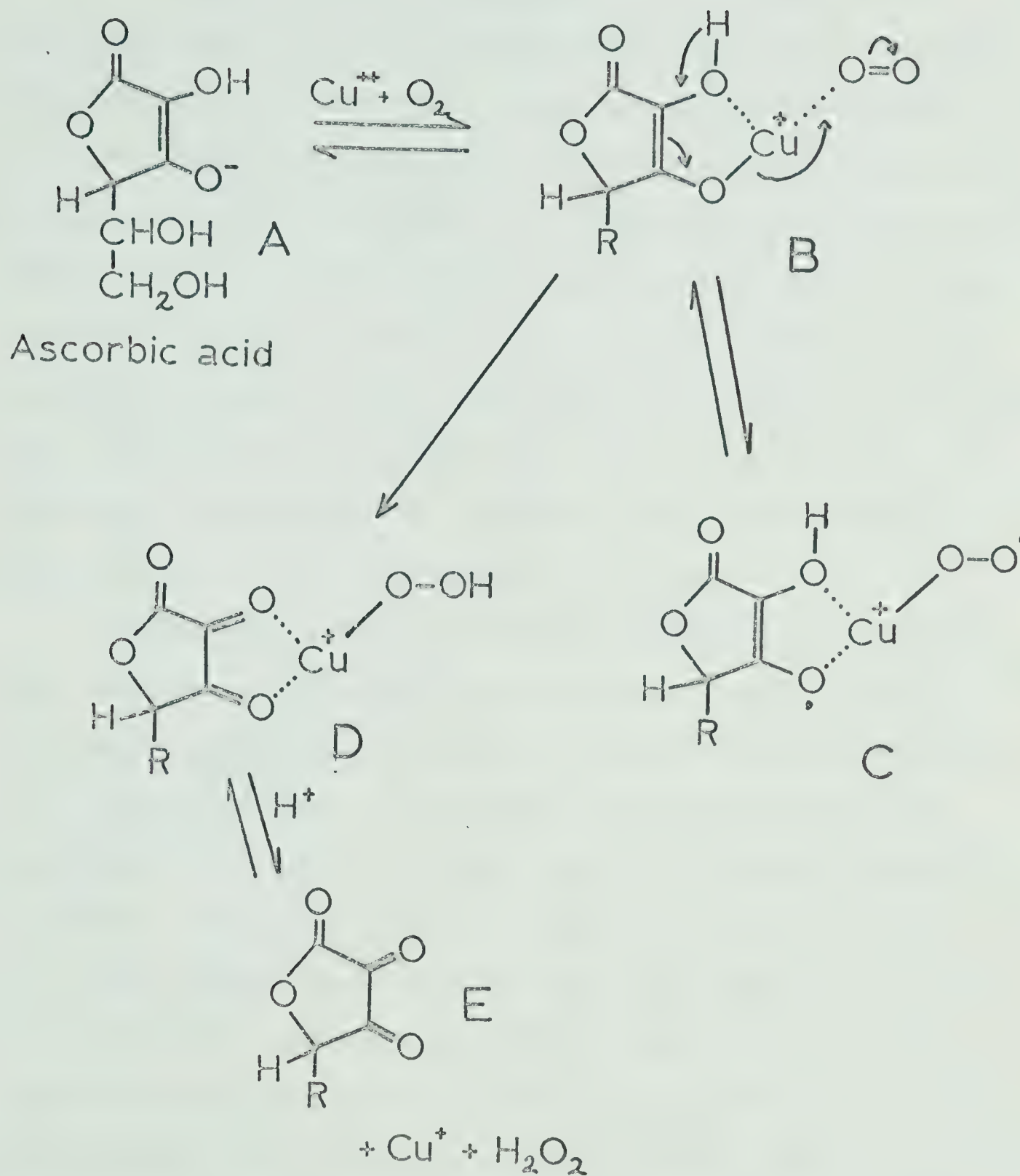


Figure 2. The mechanism of non-enzymic degradation of ascorbic acid, proposed by Kahn and Martell (1969).

EDTA, hydroxyethylenediaminetriacetic acid and iminodiacetic acid.

In all these cases, the rate of oxidation does not depend on the oxygen concentration. Also when chelating agents are present, the oxygen is reduced to water, whereas hydrogen peroxide is the reduction product in the absence of chelating agents. The mechanism offered by Kahn and Martell (1967b) as an explanation for this is quite different to that illustrated in Figure 2. The reaction catalysed by copper occurs more rapidly than the reaction which takes place in the presence of chelating agents. This appears only possible when there is a free binding site on the metal ascorbate complex for binding oxygen; when the chelating agent is present this is not available.

The peroxide content of the system discussed above was found by Harper, Morton and Rolfe (1969) to remain constant at about $0.5 \times 10^{-4} \text{ M}$ (expressed as hydrogen peroxide) after an initial maximum irrespective of the amount of ascorbic acid oxidized. Uri (1961) proposed that reoxidation of the cuprous ion absorbs some of the peroxide (Figure 2). The hydroxyl radical is available for further oxidation of ascorbic acid. Since reduction of cupric ions occurs only during initiation of the reaction, obviously this reaction can account for only a minor portion of the hydrogen peroxide produced. But hydrogen peroxide can oxidize ascorbic acid itself in the oxidation system (Harper et al., 1969). At the same time, cupric and ferric ions are capable of decomposing hydrogen peroxide with the liberation of more hydro-peroxy radicals, as follows:



c. The effect of plant pigments on the non-enzymic degradation of ascorbic acid

The pigments present in plants form a very wide and diverse group of compounds. The flavonols represent one group of pigments present in higher plants (Table 3) and members of this group such as quercetin, kaempferol, rutin and dihydroquercetin were found by Harper, Morton and Rolfe (1969) to inhibit the oxidation of ascorbic acid in the absence of added copper ions, and incubation studies have found them to be effective antioxidants in the presence of cupric ions (Clegg and Morton, 1968). Waters (1964) found the flavones to be potent inhibitors of the oxidation of oils and fats, so it is possible that a similar mechanism operates for the inhibition of ascorbic oxidation in an aqueous medium by the flavonols, since these groups of compounds are structurally related. The flavonols are structurally well suited to chain breaking reactions. Letan (1966) determined their effectiveness as antioxidants for fats and oils. Harper, Morton and Rolfe (1969) found the order of decreasing effectiveness of the flavonols, quercetin, kaempferol and rutin as antioxidants for ascorbic acid at pH 2.9 was in general the same as that for fats and oils. Dihydroquercetin was found to be as effective as quercetin. The 3,4 dihydroxy grouping is no longer conjugated with the carbonyl group and this results in a decrease in the resonance stabilization of the radical. The reason for the high activity of this dihydroxyflavonol is so far unexplained. Some of the groups of plant pigments are seen in Table 3.

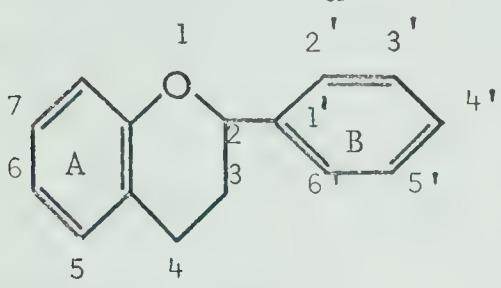
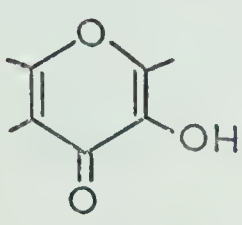
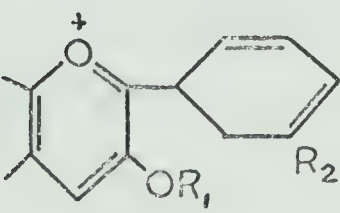
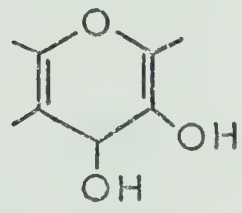
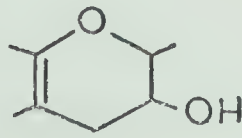
When copper ions were not present a four fold increase in the flavonoids added did not alter the amount of ascorbic acid oxidized during the four hours. When copper ions were added the rate of

oxidation of ascorbic acid fell with an increase in the quercetin concentration up to an optimum concentration of 1.25×10^{-4} M. A further increase in concentration of the antioxidant caused a reduction in the protective effect. Scott (1965) noticed this phenomenon of optimum concentration. He considered it to be due to the pro-oxidant effect of the inhibitor surpassing its antioxidant effect above a certain concentration.

Harper (1969) has studied the interaction between cupric ions and several pure flavonoids under acidic conditions such as exist in natural blackcurrant juice, i.e., pH 2.3, 3.1 and 4.5. All 3-hydroxy flavones formed complexes of comparable stability of pH 3.1 and 4.5. Only the quercetin complex was stable at pH 2.3. This is thought to be as a result of additional contributions to the resonating system arising from the presence of 4', 5 and 7 hydroxyl groups. The 5-hydroxylflavone system, seen in tectochrysin, forms unstable complexes, and this is thought to be due to a lack of any such resonating system. In this range of acidity, the 3', 4'-dihydroxyflavonoid system, including for example catechin did not form complexes with copper. Detty, Heston and Wender (1955) found stable complexes were only formed at higher pH levels.

Another group of plant pigments, the anthocyanins (Table 3) affect the non-enzymic decay of ascorbic acid. The principal anthocyanins of blackcurrant juice are cyanidin-3-rhamnoglucoside and delphinidin-3-glucoside (Morton, 1968). Clegg and Morton (1968) found that in the presence of added copper ions, the anthocyanins showed a slight protective effect. Without the addition of copper ions the oxidation of ascorbic acid was accelerated by anthocyanins.

TABLE 3

Whole Structure or Structure of the C ₃ Portion	Name of the General Class of Compounds	Typical Members and Position of Hydroxy Groups or Sugars
	All flavonoids	
	Flavonols	kaempferol; 5,7,4' quercetin; 5,7,3'4'
	Anthocyanins	pelargonidin; 5,7,4' R ₁ = H cyanidin; 5,7,3'4' R ₁ = H delphinidin; 5,7,3'4'5' R ₁ = H cyanidin-3-rhamnoglucoside R ₁ = rutinose R ₂ = H delphinidin-3-glucoside R ₁ = glucose R ₂ = H
	Flavan-3-4-diols	leucocyanidin; 5,7,3'4' leucodelphinidin; 5,7,3'4'5'
	Flavan-3-ols	catechin 5,7,3'4' galocatechin 5,7,3'4'5'

Harper (1969) has considered the anthocyanins may initiate the chain reaction in the same way as copper ions. Harper, Morton and Rolfe (1969) found that the loss of ascorbic acid was accompanied by loss of anthocyanins and this loss may be attributed to oxidation mainly by hydrogen peroxide (Jurd, 1966).

The loss of ascorbic acid due to the presence of copper ions was reduced by the flavonol, quercetin. However, in the presence of anthocyanin pigments, quercetin was less effective as an antioxidant for ascorbic acid although the effectiveness of the flavonol was increased as the concentration of anthocyanins was raised concomitantly.

This large body of information delineates the importance and the role of ascorbic acid in living systems. This study evaluates factors which cause the degradation of vitamin C in saskatoon berries, Amelanchier alnifolius, and additional inter-related factors which affect the stability of the antiscorbutic compound in one of the fruits native to Alberta.

III. METHODS AND MATERIALS

A. The Estimation of Ascorbic Acid in Saskatoon Berries and Other Fruits

Saskatoon berry plants of several varieties were cultivated at the Canada Department of Agricultural Research Station at Beaverlodge in Northern Alberta. Tests were initiated in the fall of 1969 into the frozen berries and were continued to the fresh berries of 1970. The berries were handpicked and sent immediately to the University of Alberta, Department of Food Science in Edmonton, for analysis. Several varieties of saskatoons are grown in Beaverlodge, although only a few types were actually used. Investigations were generally limited to the Smoky variety. A similar dark red variety denoted as 5003 were available as well as another denoted as 0006 which was a pale pink coloured berry. Paleface berries, which were white fruit grown from shrubs which had recently been propagated, were also used in the study.

Sunkist valencia oranges and Alberta netted-gem potatoes, purchased from a local grocer, were also used for the evaluation of assay techniques.

(1) Colourimetric techniques

The classical assay of ascorbic acid in foodstuffs or biological material is that of Tillmans (1927), where the vitamin is titrated with 2,6-dichlorophenolindophenol (DCPIP). This is a blue dye in neutral or alkaline solutions while its solutions in acid are pink and reduction leads to a colourless "leuco" compound. The reaction is given in

Figure 3. The DCPIP solutions were prepared by dissolving tablets containing the dye in warm deionised water, filtering the solution through a Whatman No. 1 filter paper and diluting to known volume. This was stored at 4°C and the oxidising power of the dye was checked regularly. Fresh solutions were prepared weekly and standardised against an aqueous solution of ascorbic acid of known concentration.

Saskatoon berries (10 gm) were ground thoroughly in deionised water (50 ml) and the pulp was passed through a 5 μ millipore filter. Aliquots of the diluted juice (20 ml) were acidified with oxalic acid (1.5 g) and the solution was titrated with the standardised DCPIP, from a microburette.

The end point of the titration is pink and as the colour of the saskatoon juice is also pink the colour change of the dye was impossible to detect. The basic method described above was modified in the following ways to attempt to overcome this problem:

(a) Diethyl ether (10 ml) was added to extract the DCPIP when the end point was near and the colour change of the ether layer was noted.

(b) The pink pigments present in saskatoon extracts were removed by absorption on activated charcoal. Other methods used to achieve this removal were extraction of the pigments in a mixture of hydrochloric acid and methanol, and extraction of a trichloroacetic acid extract with iso-amyl alcohol.

(c) The indophenol-xylene extraction method of Robinson and Stotz (1945) was used. This involved measuring the colour produced by the DCPIP spectrophotometrically at 500 m μ .

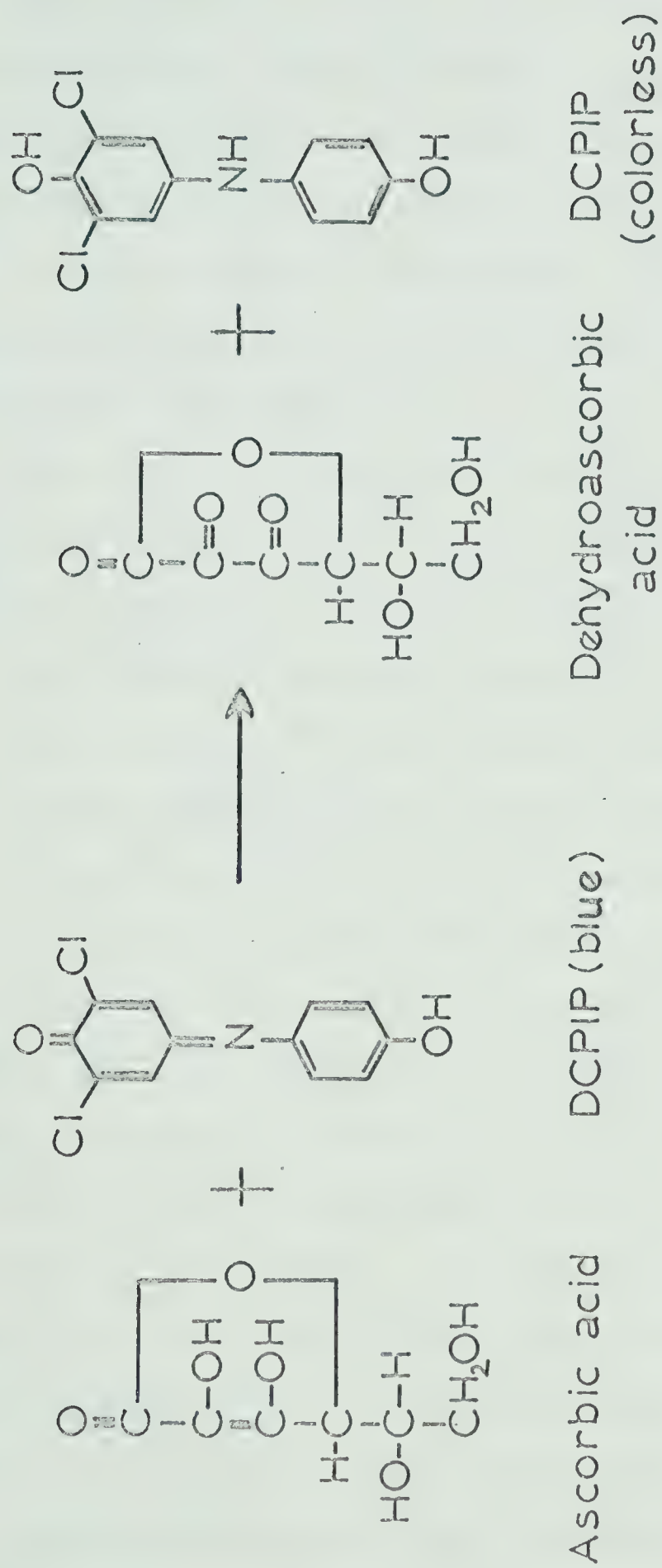


Figure 3. The reaction of ascorbic acid with 2,6-dichlorophenolindophenol in the colourimetric assay of ascorbic acid.

(2) The polarographic method

The polarographic method of ascorbic acid assay, following the work of Brezina-Zuman (1956) as modified by Wasa, Takagi and Ono (1961) was found to be both simple and accurate. An acetate buffer, pH 5.5, with 1% formaldehyde added was saturated with sodium oxalate and filtered through a Whatman No. 1 filter paper. The filtrate is used as the supporting electrolyte, and in this medium the half wave potential of ascorbic acid is about +0.07 volt.

Ten grams of saskatoon berries were ground thoroughly at 0°C with 50 ml of deionised water. Ten ml of this diluted juice was added to an equal volume of the supporting acetate electrolyte and the total solution was purged with nitrogen (99% purity) for four minutes in an 'H' type polarographic cell. The dropping mercury cathode was then placed into the cell and the anodic current was applied from -0.1 → +0.1 volts employing a Sergant Model XV Polarograph. Details of the cell design can be seen in Figure 4. From the polarographic trace obtained, the wave height was determined and this in turn was converted by means of a calibration curve (Figure 5) into an amount of ascorbic acid in microgram quantities per millilitre of polarographed solution. The calibration curve was attained using a series of standard ascorbic acid concentrations, ranging from 0 - 75 µg/ml of synthetic L-ascorbic acid (Figure 5). Over this range of concentrations there was a linear relationship between ascorbic acid present in solution and wave height, as measured on the polarographic trace (Appendix I).

In this polarographic method, measurement of the diffusion current--i.e., the wave height reproduced on the polarogram, which is

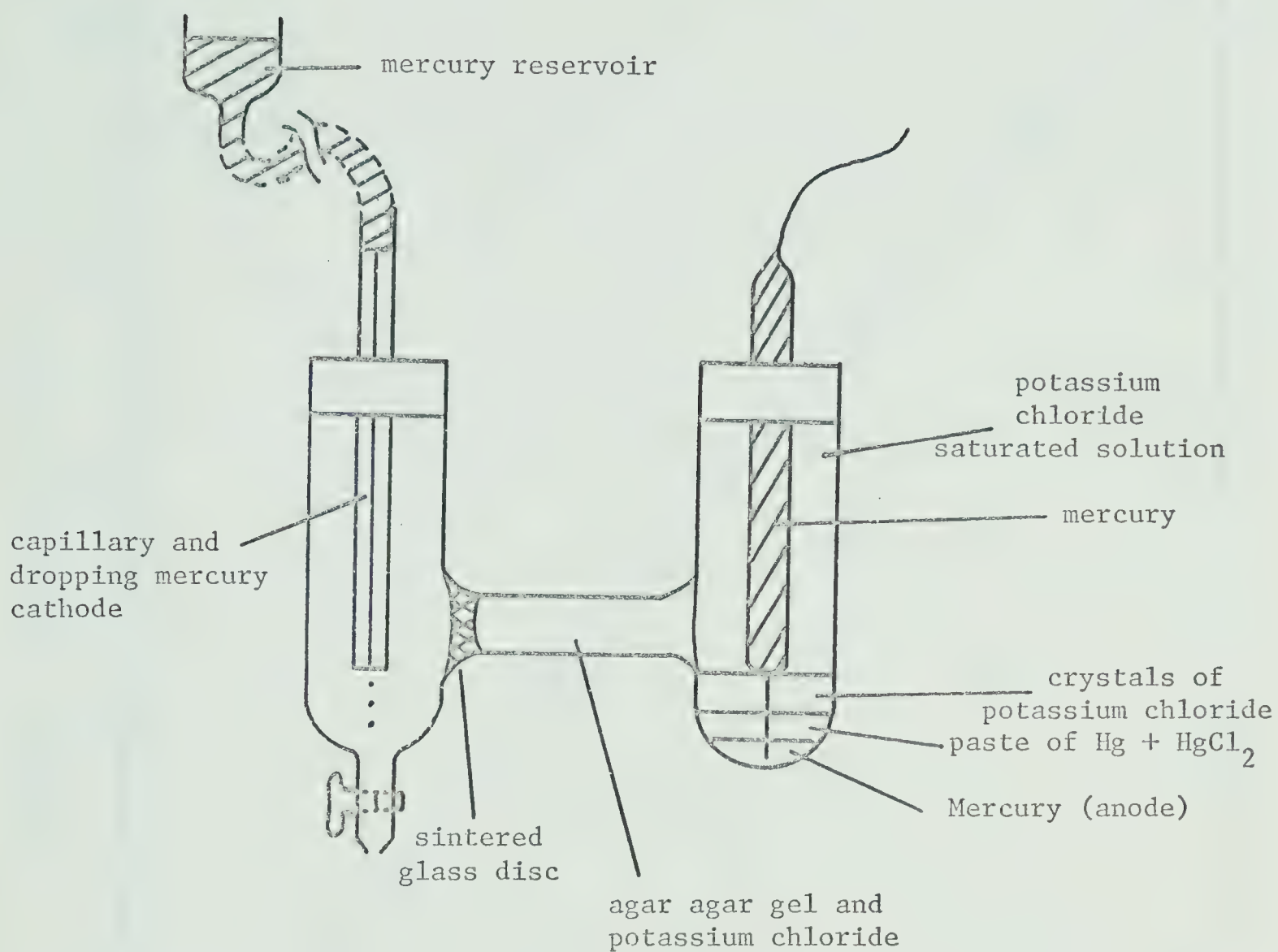


Figure 4. Diagram of the dropping electrode assembly used in the polarographic assay of ascorbic acid.

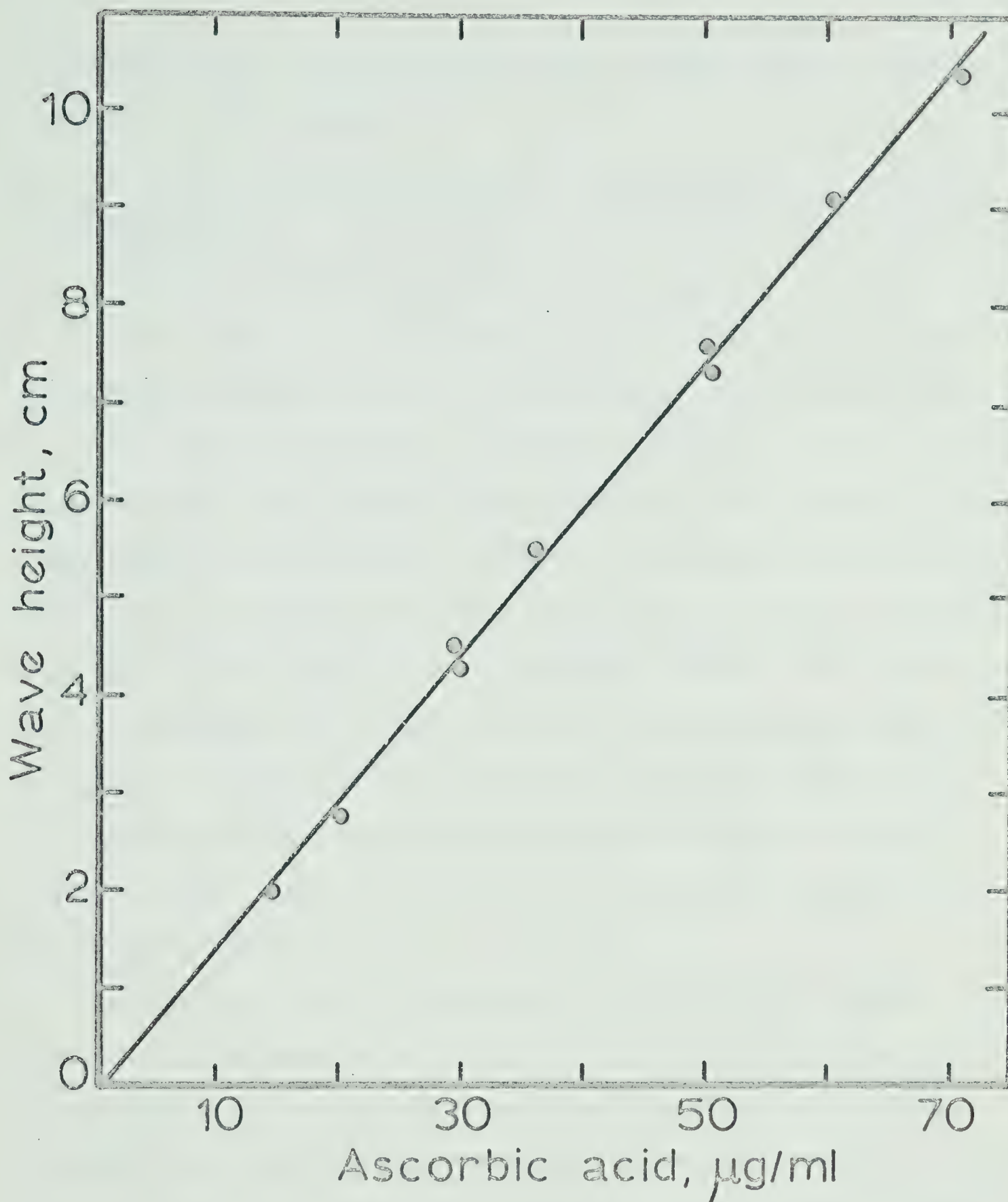


Figure 5. Calibration curve of polarographic wave height versus concentration of ascorbic acid.

indicative of the concentration of ascorbic acid was done by extrapolation as indicated in Appendix I.

B. Techniques Used for the Estimation of Dehydroascorbic Acid in Saskatoon Berries

(1) Reduction method with 2,3-dimercaptopropanol

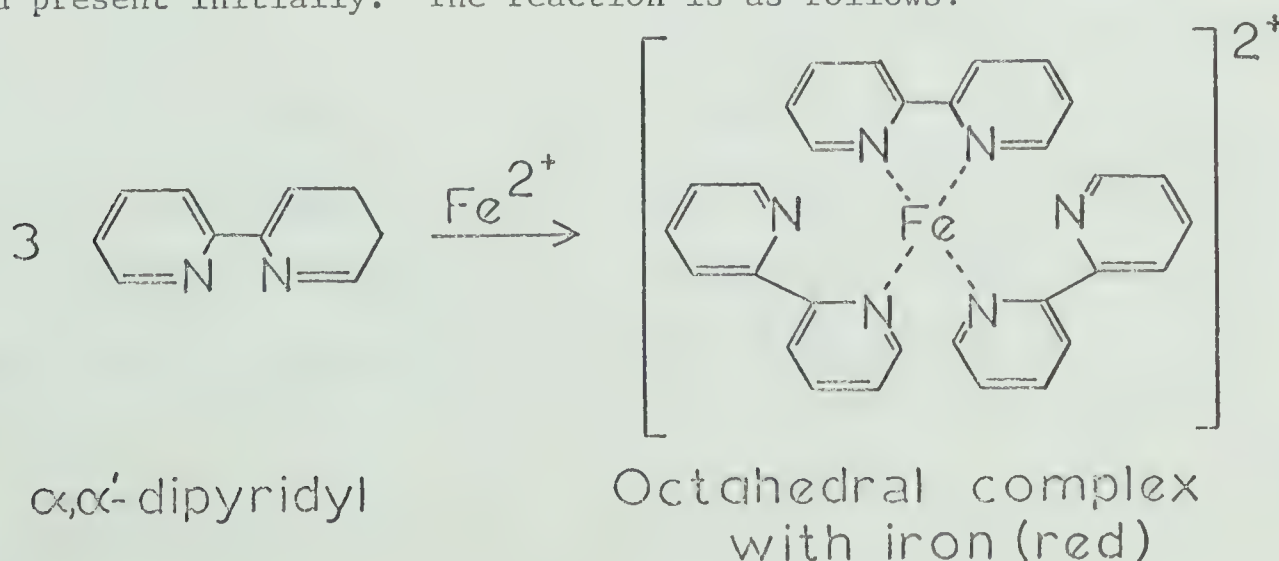
The method of Gero and Candido (1969) was used for the estimation of dehydroascorbic acid. The reducing agent which had been used for a long time in this context was hydrogen sulphide. Apart from the inconvenience of the technique, King (1941) found this to be not in the least specific and the method led to a number of false and doubtful results. Peters, Stocken and Thompson (1945) discovered during the last war that 2,3-dimercaptopropanol or British Anti Lewisite (BAL) could be used as an antidote for certain war gases. These workers also found that BAL was capable of reducing dehydroascorbic acid. The fact that 2,3-dimercaptopropanol in slight excess reduces dehydroascorbic acid rapidly and quantitatively at pH 6.8 makes BAL an ideal compound for the reducing procedure.

Five grams of fruit were homogenised with 20 ml of 4% trichloroacetic acid/2% metaphosphoric acid pH 2.4. After centrifugation (10 min at 7,000 x g) the supernatant was made up to 20 ml with the acid mixture. Two aliquots of 2 ml were placed in centrifuge tubes in an ice bath, and were neutralised to pH 6.8 with saturated potassium monophosphate. Aqueous 2,3-dimercaptopropanol (0.2 ml of 0.1 M) is placed into the first tube and an equal quantity of water is added to the blank. The first tube is removed to a thermostated bath at 30°C while the control remains in the ice bath. After 10 minutes, 0.4 ml of 2 M cadmium

chloride is added to contents of the two tubes which are then mixed with 2.6 ml of distilled water and centrifuged for 4 minutes at 7,000 x g. The supernatants obtained were analysed immediately on the polarograph using 10 ml of the acetate supporting electrolyte as described previously. The total vitamin C content--i.e., the L-ascorbic acid plus dehydroascorbic acid was found from the calibration curve (Figure 5). The dehydroascorbic acid content of the berries was then found by subtracting the value obtained for the control tube (ascorbic acid) from the value obtained from the solution which had been treated with 2,3-dimercaptopropanol (representing L-ascorbic acid and dehydroascorbic acid).

(2) Titration of ascorbic acid and dehydroascorbic acid with α, α' -dipyridyl

The second method used for the determination of dehydroascorbic acid in saskatoon berries was the colourimetric assay of Baczyk and Karlik (1969). This method involves the reduction of dehydroascorbic acid to L-ascorbic acid using sodium sulphide. The ascorbic acid formed is treated with ferric chloride and the extent of the reduction of ferric ion is estimated with the use of α, α' -dipyridyl. Since ferrous ion and α, α' -dipyridyl form a coloured complex, the spectrophotometric measurement of the colour density is therefore an index of the dehydroascorbic acid present initially. The reaction is as follows:



Ten grams of saskatoon berries were homogenised, and the juice obtained by centrifugation at $2,500 \times g$ plus 0.5 ml of glacial acetic acid was made up to 100 ml with water. After extraction for 10 minutes, a 10 ml aliquot of the solution was added to an equal volume of 1N hydrochloric acid and 1.4 ml of disodium sulphide. After 15 minutes, 3 ml silver nitrate (2 M) were added to precipitate the excess sulphide. The volume of the aliquot was made up to a total volume of 50 ml, and then filtered through a Whatman No. 1 filter paper.

The ascorbic acid content of this solution was measured by mixing 2 ml of filtrate with 0.25 ml 10% orthophosphoric acid, 1 ml 0.5% α, α' -dipyridyl solution and 0.1 ml 1% FeCl_3 . After 30 minutes distilled water was added to a volume of 10 ml, and the absorbance was read at approximately 520 m μ on a Spectronic 20. The blank determination was conducted without the addition of α, α' -dipyridyl solution.

A calibration curve was made (Figure 6) from values obtained using solutions containing 0 -- 25 $\mu\text{g/ml}$ ascorbic acid, and from the spectrophotometric values obtained from the saskatoon filtrate the ascorbic acid and dehydroascorbic acid content present in the berries was determined.

C. The Degradation of Ascorbic Acid by Saskatoon Berry Juice

Samples of berries (15 g) were homogenised in a Waring Blendor at 0°C for two minutes and the juice obtained by passing the mulch through a centrifugal juice maker was made up to 50 ml with water. An aqueous solution of ascorbic acid, containing 100 $\mu\text{g/ml}$ was added to the berry extract to bring the total volume to 500 ml.

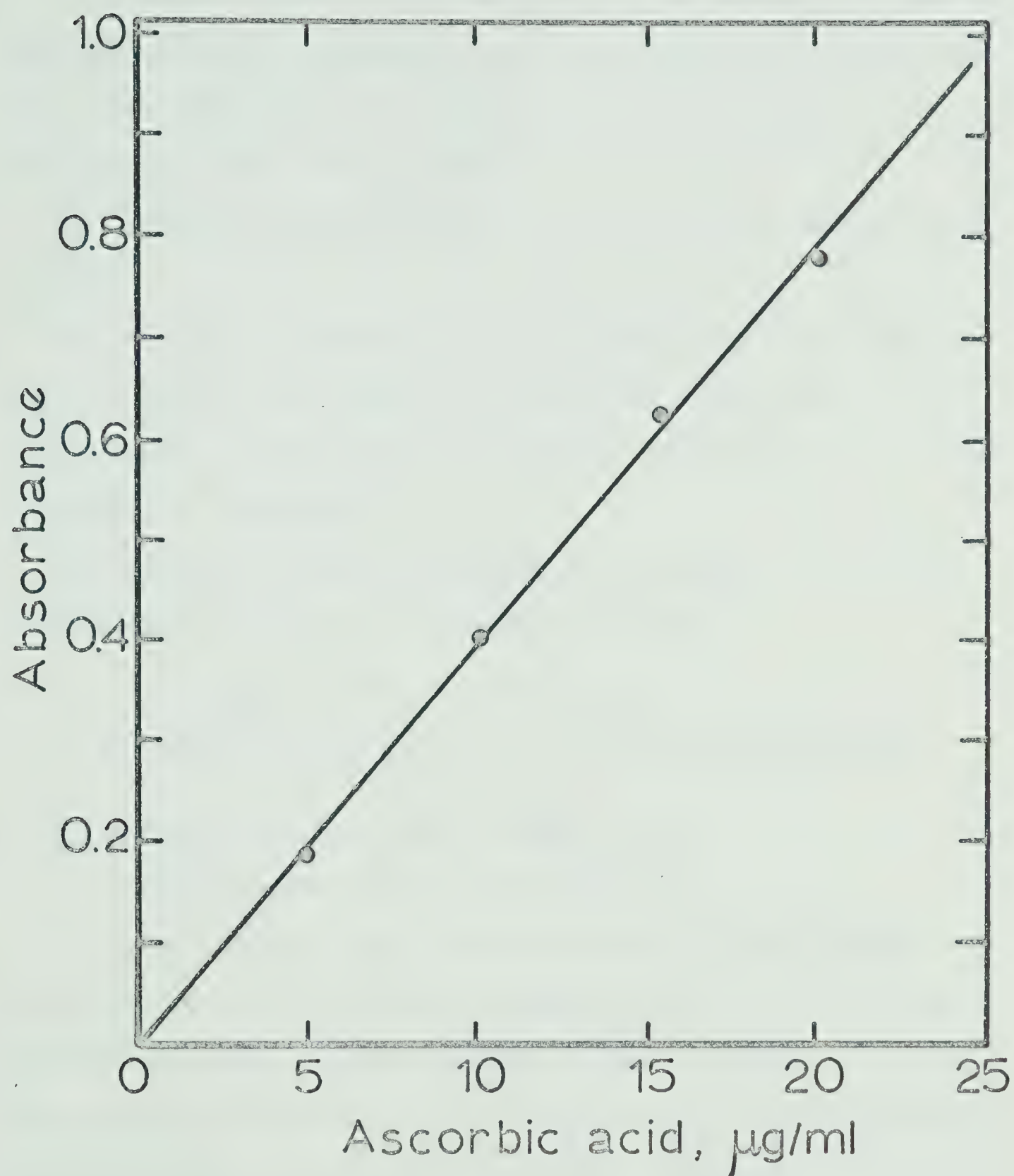


Figure 6. Calibration curve for the estimation of ascorbic acid and dehydroascorbic acid using α, α' -dipyridyl.

A sample of the standard ascorbic acid was assayed polarographically and assays were continued for 80 - 100 minutes after the acid had been added to the juice. Variations of this "basic" experimental method were applied to the following samples:

(1) Saskatoon berries harvested at various stages of maturity were analysed.

(2) The decay of ascorbic acid was observed when it was added to the juice obtained from Smoky berries which had been stored at -12°C for six months in sealed plastic containers, to see the effects, if any, of freezing on the berries.

(3) Each juice extract was treated for 20 minutes in water baths of the following temperatures (degrees Centigrade):

0° , 20° , 30° , 40° , 50° , 98°

before addition of the berry juice to the ascorbic acid solution.

D. The Activity of Ascorbic Acid Oxidase

(1) Crude ammonium sulphate precipitation

Smoky berries (15 gm) were homogenised in a Waring Blendor for two minutes in a citrate phosphate buffer (75 mls), pH 6.6, prepared according to Porath, Samorodova-Bianki and Hjerten (1967). The juice was separated from pieces of skin and pips present in the berries by a small centrifugal rotor and was subsequently centrifuged at $900 \times g$ in a Beckman Model J21 refrigerated centrifuge. The juice so obtained was held at 0°C and 60 gm of solid ammonium sulphate was added. This solution was held for 15 minutes in ice with occasional shaking and then centrifuged at $2,500 \times g$. The precipitate was retained, and 30 grams of

solid ammonium sulphate was added to the supernatant. After holding for a further fifteen minutes in ice, this solution was centrifuged at $6,300 \times g$. A third precipitate was obtained in similar fashion, by adding more of the solid salt (10 gm) to the supernatant of the second centrifugation step.

The second and third precipitates were pooled and dissolved in an aqueous solution which contained $100 \mu\text{g/ml}$ ascorbic acid. Samples of this stock solution (10 ml) and equal quantities of the acetate supporting electrolyte were taken and immediately analysed polarographically as described in Part 1 of this section.

This same procedure was followed for the supernatant of the extractions.

(2) The preparation of a more sophisticated ammonium sulphate precipitate

Dawson and Magee (1955) used a purification procedure for ascorbic acid oxidase present in the squash plant Cucurbita pepo. This same method was used with saskatoon berries to the stage of refractionation with ammonium sulphate.

The juice was extracted from Smoky berries (250 gm) by homogenisation in a Waring Blendor and subsequently centrifuged at $2,000 \times g$ in a Beckman Model J21 refrigerated centrifuge. Solid sodium bromate was added to the juice to bring the pH to 7.6. At room temperature, the crude juice was treated with 5 ml of barium acetate (1 M) and 200 gm of solid ammonium sulphate.

The precipitate was allowed to settle overnight at 4°C at which point the supernatant fluid was removable almost entirely by siphon.

The precipitate was discarded and the supernatant was treated with a further 200 gm of ammonium sulphate. The resulting precipitate was redissolved in 500 ml of cold deionised water. After filtration of the redissolved precipitate, the resulting solution was dialysed against several changes of distilled water at 4°C for 36 hours. A pale pink solution was obtained and a sample of this equivalent to 100 gm of berries was used for the following analysis. This solution, to which ascorbic acid was added (100 µg/ml), was made up to volume with a phosphate/citrate buffer (pH 6.6) according to Porath, et al. (1967). Aliquots of the ascorbic acid solution (10 ml) were analysed on the polarograph before and after the addition of the dialysed sample, so the decay of ascorbic acid induced by the saskatoon berry extract could be found.

(3) Removal of microorganisms

Sodium hypochlorite (5% aqueous solution) was found to be an effective surface sterilant for the removal of microbial contaminants from the Smoky berries. The effectiveness of the procedure used was found in the following way: five grams of berries were washed in 10 ml of sterilised water and 1 ml samples of the washings were plated onto potato dextrose agar containing tartaric acid (1 ml of 1% solution for each plate) and also plate count agar. A similar sample of berries were treated for one minute in the hypochlorite solution, washed three times in sterilised water and then plated out onto the two kinds of agar.

The growth of colonies of both fungi and bacteria on potato dextrose agar and plate count agar was reduced by the surface sterilising procedure. This is especially the case with berry washings plated on the plate count agar. An average of 20 colonies were found in the un-

treated sample and these numbers were reduced to 3 after treatment in the hypochlorite. Investigations into the bacterial and fungal types was thought to be unwarranted, because assuming one bacteria gives rise to one colony, the degree of contamination--of the order of 40/gm of fresh berry, is negligible.

With the efficiency of this sterilising technique established, saskatoon berries (15 gm) were sterilised and washed three times in de-ionised water. They were later pulped and to the juice was added ascorbic acid using the same procedure as outlined in Part A of this section--the decay of added ascorbic acid was followed polarographically.

E. The Effect of Copper Ions and Phenolic Compounds on the Saskatoon Juice System

(1) Copper estimation

An Unicam atomic absorption spectrophotometer (S.P.-90) was used to measure the copper content of the saskatoon berry juice at a wavelength of 324.8 mμ and slit width 0.08 mm. A 10 cm propane burner was used with a burner height of 1.2 cm. The fuel source was propane, at a pressure of 10 p.s.i. and flow rate of 300 cc/min. The pressure of air used was 30 p.s.i. at a flow rate of 5 l/min.

A stock copper solution was made up by dissolving pure copper metal in 2 - 3 ml of 25% hydrochloric acid/nitric acid and making up to one litre with water.

The instrument was calibrated using solutions containing copper (0 - 15 ppm) and a graph was plotted of copper concentration against absorbance (Figure 7). The soluble components of 10 gm samples of saskatoon berries were extracted with 4% metaphosphoric acid/2% trichloroacetic

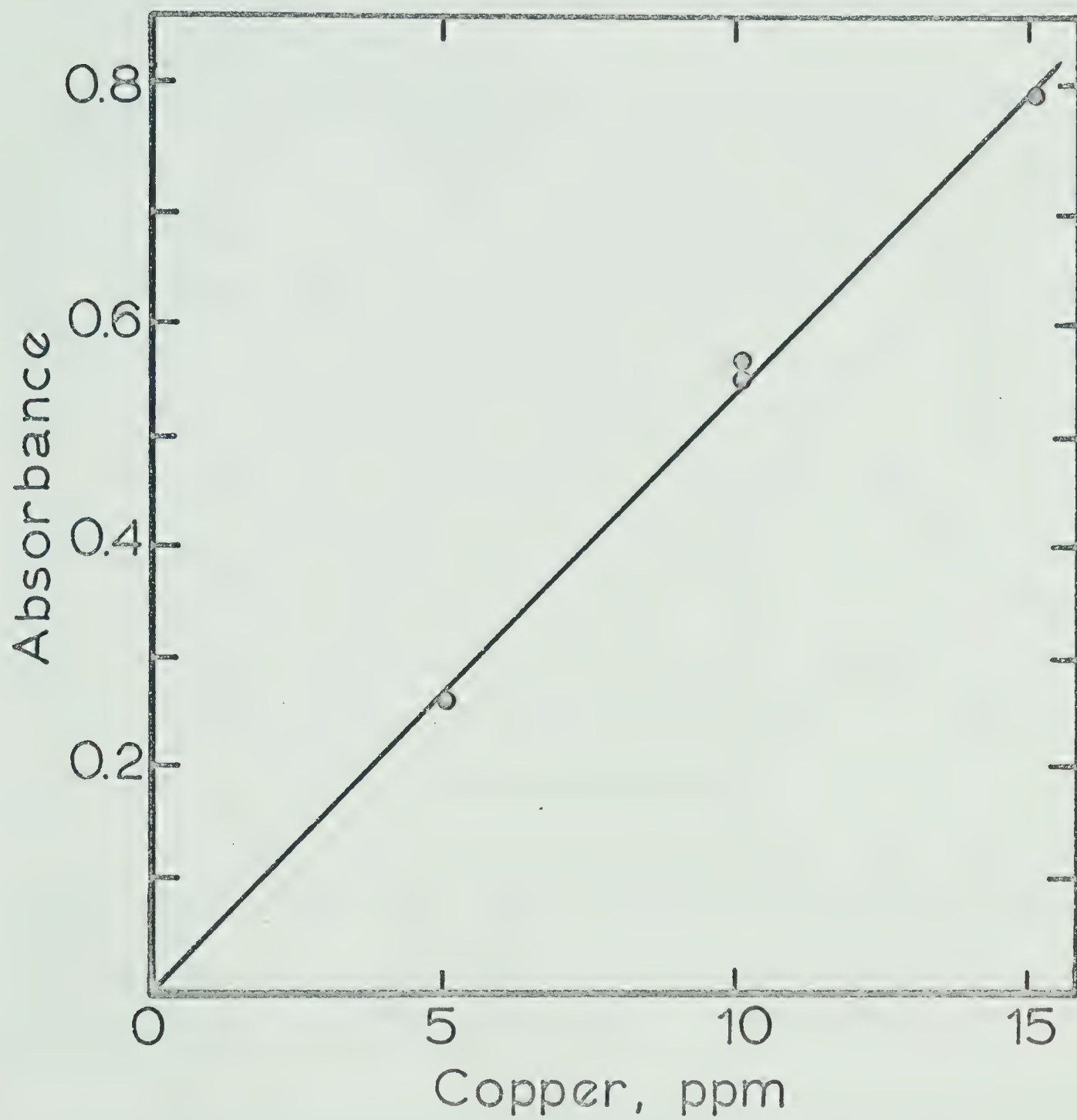


Figure 7. Calibration curve of the Unicam atomic absorption spectrophotometer S.P.-90, using standard copper solutions.

acid pH 2.4 and the absorbance was measured at 324.8 mμ. The copper content of the berries was calculated from the calibration curve.

- (2) The effect of copper on the decay of ascorbic acid when added to saskatoon juice

The slurry obtained by homogenising both Smoky and Paleface berries (15 gm) with citrate/phosphate buffer pH 6.6 (Porath, et al. 1967) was centrifuged at 7,000 x g on a Beckman Model J21 refrigerated centrifuge. The juice obtained was made up to volume (500 ml) with added ascorbic acid (0.05 gm) and copper sulphate. Copper was added to give six final concentrations as follows: 10, 25, 50, 75, 100 and 200 ppm.

For each copper concentration, after thorough mixing of the solutions together, 10 ml aliquots were analysed on the polarograph (with 10 ml of supporting acetate electrolyte) under the conditions outlined in Figure 4, Figure 5 and Appendix I. The assay was completed over a 90 minute period and the decay of ascorbic acid with time was determined and expressed after one hour as explained previously.

An identical experiment was also carried out for the Smoky and Paleface berries, but in this case the extraction medium was a solution of 4% metaphosphoric acid/2% trichloroacetic acid (v/v) of pH 2.4 as suggested by Barker and Mapson (1951).

- (3) The effect of varying concentrations of saskatoon juice on the decay of ascorbic acid in the presence of copper ions

In these experiments the amount of saskatoon juice is the variable factor. The concentration of copper ions added to the citrate/phosphate buffer, pH 6.6 was constant at 10 ppm. The final volume of solution was 500 ml, and in addition to the buffer and copper present,

0.05 gm of ascorbic acid was added (i.e., 100 µg/ml). Juice was present in the system also; this had been extracted with buffer and added to the buffer/copper/ascorbic mixture in such a way so that 10 ml aliquots of the total reaction mixture contained juice equivalent to 1.0 gm of fresh saskatoons. As before, ascorbic acid was assayed polarographically for a 90 minute period. These experiments were repeated varying the concentration of saskatoon juice so that the total reaction solution contained juice equivalent to 0.25, 0.5 and 1.5 gm of berries. The complete method were repeated for the Paleface fruit obtained from the fourth harvest.

An identical series of experiments were carried out with the citrate/phosphate buffer (pH 6.6) extraction replaced by a metaphosphoric acid/trichloroacetic acid of pH 2.4 extraction for both the Smoky and Paleface berries.

(4) The effect of phenolic components on saskatoon juice extracts

Fifteen gram samples of Smoky and Paleface berries (harvested on July 30, 1970) were homogenised in a 4% metaphosphoric acid/2% trichloroacetic acid buffer of pH 2.4 (Barker and Mapson, 1951). The juice obtained by centrifugation of 2,500 x g was made up to volume (500 ml) with the buffer. Ascorbic acid was added to this (100 µg/ml) and at concentrations of 1.0×10^{-4} M: peonin, delphinidin, malvin, quercetin, rutin, catechol and kaempferol. The content of ascorbic acid in the system was detected polarographically, as described in part A of this section, Figure 4, Figure 5 and Appendix I, to observe the effect(s), if any, of each phenolic compound.

IV. RESULTS

A. The Estimation of L-Ascorbic Acid in Saskatoon Berries and Other Fruits

(1) The colourimetric methods

The classical methods of ascorbic acid assay, involving the use of dichlorophenol-indophenol gave negative results for the Smoky and Paleface saskatoons. Even removal of the pigments found in the Smoky berries by the various means of extraction used proved fruitless. The indophenol-xylene extraction method of Robinson and Stotz (1945) which had been used previously for the estimation of ascorbic acid in fruit, showed no ascorbic acid present in the saskatoons at all. The methods were checked using fresh orange juice, squeezed from Sunkist oranges and the juice of a potato homogenate. Solutions of dye were made up regularly and standardised against freshly prepared ascorbic acid solutions of known concentration. In most cases 1 mg/ml of L-ascorbic acid solution was more or less equivalent to 1 ml of the dichlorophenol-indophenol dye. The values obtained on oranges and potatoes were relatively consistent and correlated well with published data. The ascorbic acid content of the orange juice was found to be 62.5 mg/100 ml juice and for the potato juice the content found was 13 mg/100 gm on a fresh weight basis. The value for potatoes is within the limits given by Sweeney, Hepner and Libeck (1969) in Table 2.

In all cases, the appearance of the end-point, that is, a pale pink colour which was permanent for three seconds was difficult to detect exactly.

(2) The polarographic method

The absence of ascorbic acid in saskatoon berries as found by the titration method was confirmed by the polarographic method. This was a swifter and more reliable method and produced results of far greater precision and accuracy for the ascorbic acid content of orange juice and potatoes. The calibration curve given in Figure 5 and Appendix I represents a typical polarographic trace, as described previously. When the method was repeated for saskatoon berries only the wave produced by the supporting electrolyte was apparent.

At concentrations of between 25 and 50 μg ascorbic acid/ml as detected by the polarograph over these concentrations, which will result in a variation of $\pm 2-3\%$ in the raw data.

B. The Estimation of Dehydroascorbic Acid in Saskatoon Berries

(1) Reduction method with 2,3-dimercaptopropanol

The modified method of Gero and Candido (1969) showed there was more than a negligible quantity of dehydroascorbic acid in the Smoky fruit. From the results of at least two experiments the average wave height was calculated back to a value of 23.9 mg dehydroascorbic acid per 100 gm (fresh weight).

Since the control tube, untreated with 2,3-dimercaptopropanol, gave negative results the value obtained represents the total vitamin C content of the berries and confirms the previous findings that saskatoons are devoid of L-ascorbic acid.

(2) Estimation of ascorbic acid and dehydroascorbic acid with α, α' -dipyridyl

Since 2,3-dimercaptopropanol tended to be somewhat noxious,

the colourimetric assay of Baczyk and Karlik (1970) was employed.

Figure 6 represents the calibration curve of absorbance versus ascorbic acid concentrations (0 - 25 $\mu\text{g/ml}$). The 2 ml of filtrate added to the final α, α' -dipyridyl solution contained the equivalent of 0.004 gm of fresh berries. The absorbance reading obtained was the equivalent of 5.47 μg ascorbic acid/ml from the calibration curve, or a total of 21.9 mg/100 gm berries (fresh weight).

C. The Effect of Saskatoon Berry Juice on Synthetic L-Ascorbic Acid

The decay of ascorbic acid after it has been added to the juice of mascerated saskatoon berries which were harvested in 1969 is given in Figure 8. All the results have been calculated to give values for 10 gm samples of berries on a dry weight basis. Since a dilution factor is involved the results have been recalculated to a basis of solutions which would at time zero contain exactly 50 $\mu\text{g/ml}$ of ascorbic acid. The graphs obtained in the study which follow the degradation of the vitamin are generally similar in that the greatest rate of decay is in the first 30-40 minutes after the addition of the acid to the juice fractions. After one hour of analysis, 22 $\mu\text{g/ml}$ of ascorbic acid had been degraded. This represents 80% of the total degradation which occurred. The results of two separate experiments are pooled and finally expressed in the form of a histogram, representing the percentage of ascorbic acid which remained after 1 hour, as illustrated in Figure 8.

The possibility of the autoxidation of ascorbic acid itself in the system cannot be ignored, so to minimise error which could occur, the oxidation of aqueous ascorbic acid was followed and the results

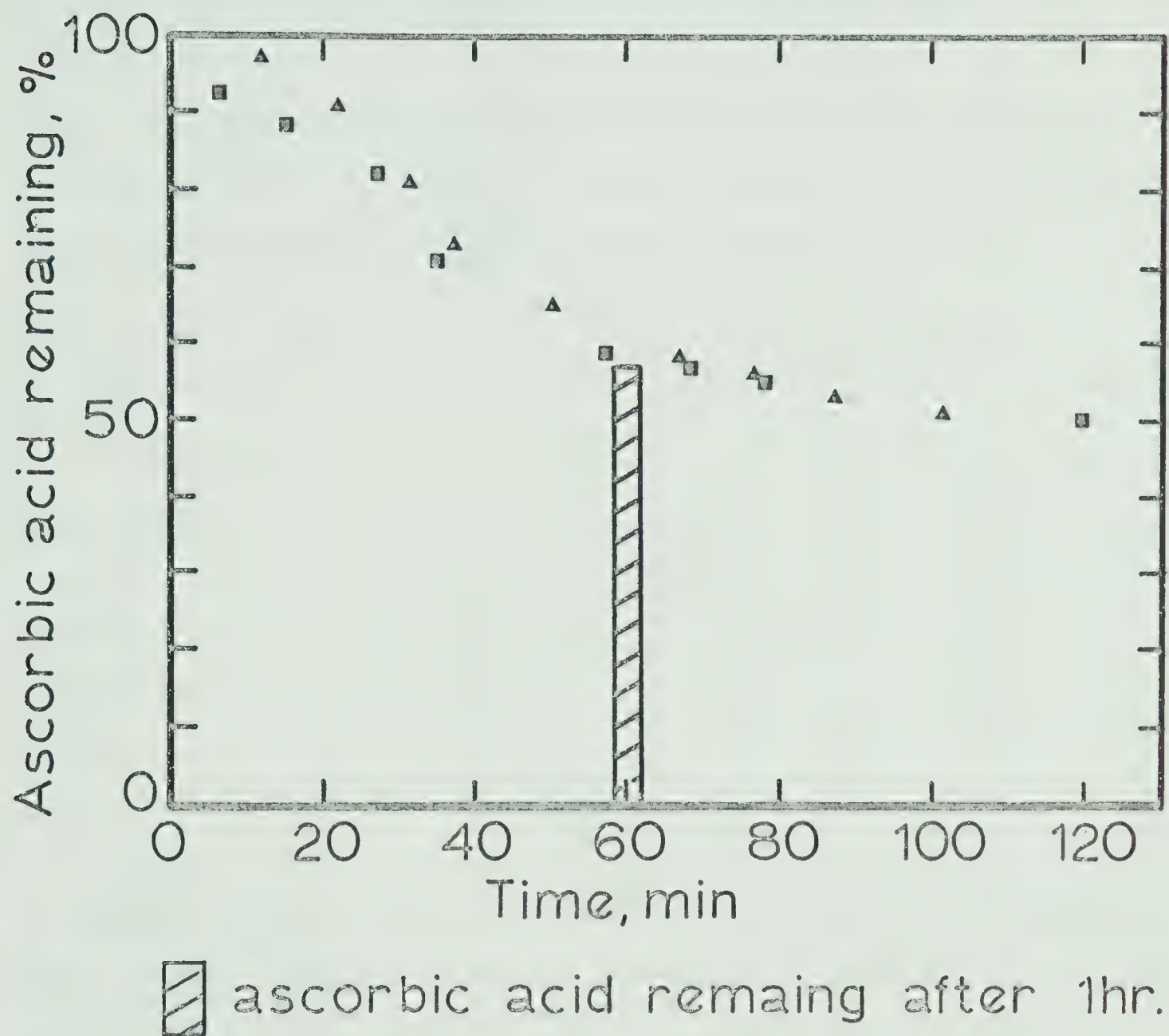


Figure 8. The effect of saskatoon berry juice on the degradation of synthetic ascorbic acid and a histogram to show the ascorbic acid (%) remaining after one hour in the system.

corrected to allow for this decrease. The histogram merely represents the decay of ascorbic acid actually induced by the saskatoon juice. The variations of berry samples used in this experiment gave results as follows:

(1) Saskatoon berries which had been harvested in 1970 were analysed to observe the effect of advancing maturity on the ability to degrade ascorbic acid. Samples received are listed below.

Harvest time	8 July	17 July	24 July	30 July	6 Aug.
Batch no.	1	2	3	4	5
Berry type					
Smoky	✓	✓	✓	✓	✓
Paleface		✓	✓	✓	✓
0006			✓	✓	✓
5003	✓	✓		✓	✓

It should be noted at this point that the fourth batch of berries--i.e., those received from Beaverlodge and analysed on 30 July 1970 had reached full maturity. The quality of fruit on 6th August 1970 had deteriorated and many berries were overripe. The berries were still immature and unripe for the first three harvests, so unless otherwise stated, the fourth batch of saskatoons harvested in late July 1970, were used in subsequent experiments.

The effect of approaching maturity on the decay curve can be seen in Figures 9 and 10. Batches of berries harvested on the different dates are compared separately (Figure 9) and then the varieties

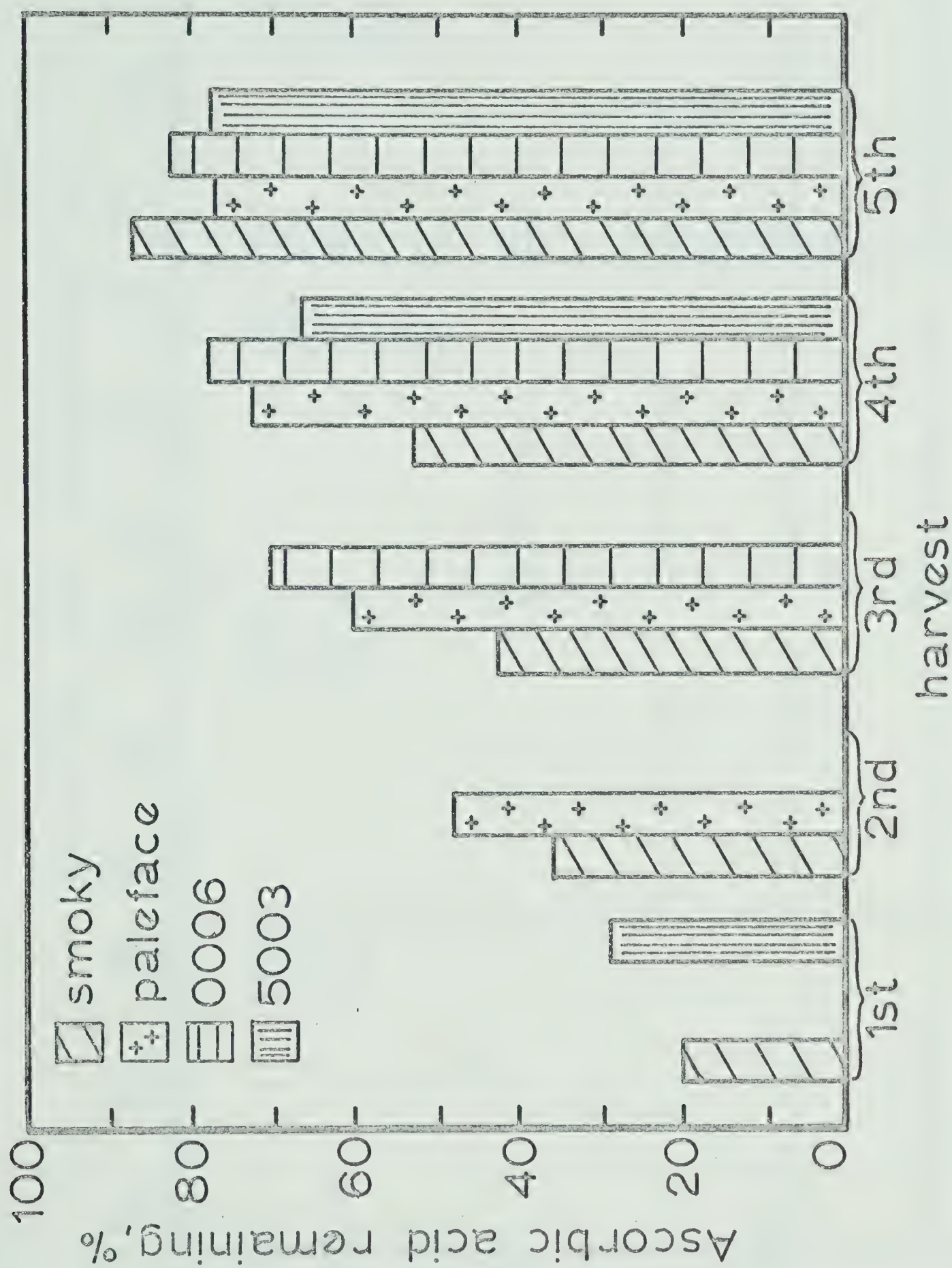


Figure 9. A comparison of the decay of ascorbic acid by four different saskatoon berry varieties, obtained from five harvests expressed as the amount of ascorbic acid (%) remaining after one hour. Harvest dates are given on page 48.

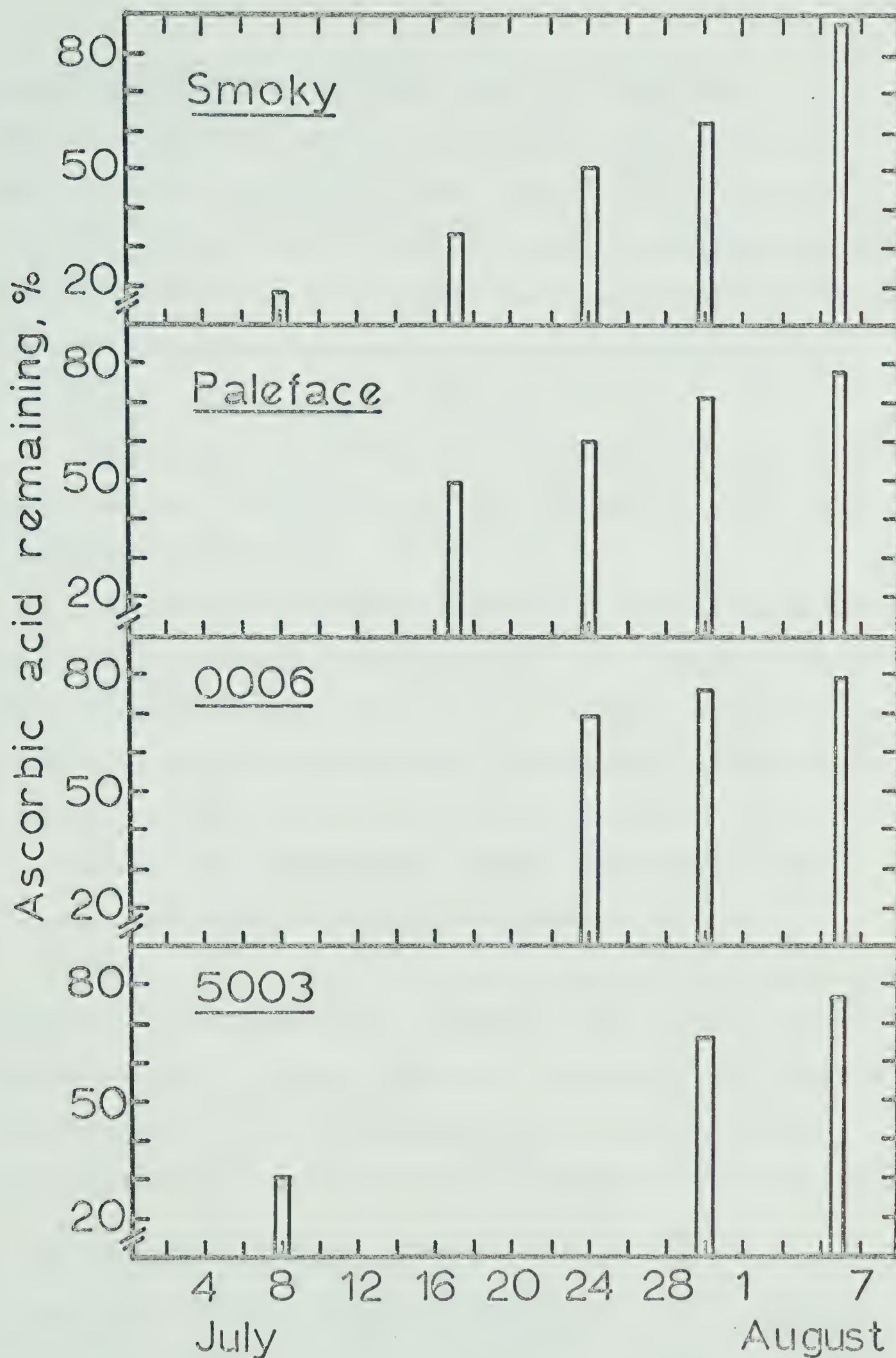


Figure 10. The effect of approaching maturity on the decay of ascorbic acid by four varieties of saskatoon berries. Each variety is compared separately on a physiological time scale.

compared separately on a time scale (Figure 10). Expression of the results on a dry weight basis is essential as the first harvest of saskatoons in all varieties consisted of tiny, hard green berries unlike the succulent fruit of the fourth harvest. Berries were placed in an oven, and after 72 hours at 65°C, the dry weights obtained for all four varieties analysed were constant in the order of 68% and in a close range for each harvest, i.e., $\pm 2\%$.

These factors were considered when mixtures of berries were used for analyses in the 3rd, 4th and 5th harvests. The results are expressed in Figure 11.

(2) The effect of storage, freezing and later thawing on the saskatoon berry tissue is seen in Figure 12. For comparison, a histogram of the ascorbic acid remaining in the freshly harvested berries in 1970 is shown together with the results obtained from a similar sample of berries which had been frozen at -12°C for six months. The percentage of ascorbic acid remaining after one hour, as detected by the polarogram, was similar for both these experiments.

(3) Results of the varying temperature treatments on the saskatoon juice system is given in Figure 13. For the juice which had been boiled for 20 minutes 90% of the ascorbic acid added initially to the fruit juice remained after one hour, as opposed to the juice which was held at 40°C for this length of time where only 50% was left.

D. The Ascorbic Acid Oxidase Activity

(1) Crude ammonium sulphate precipitation

The results of preliminary investigations designed to determine the effects of the crude ammonium sulphate fractionation on the de-

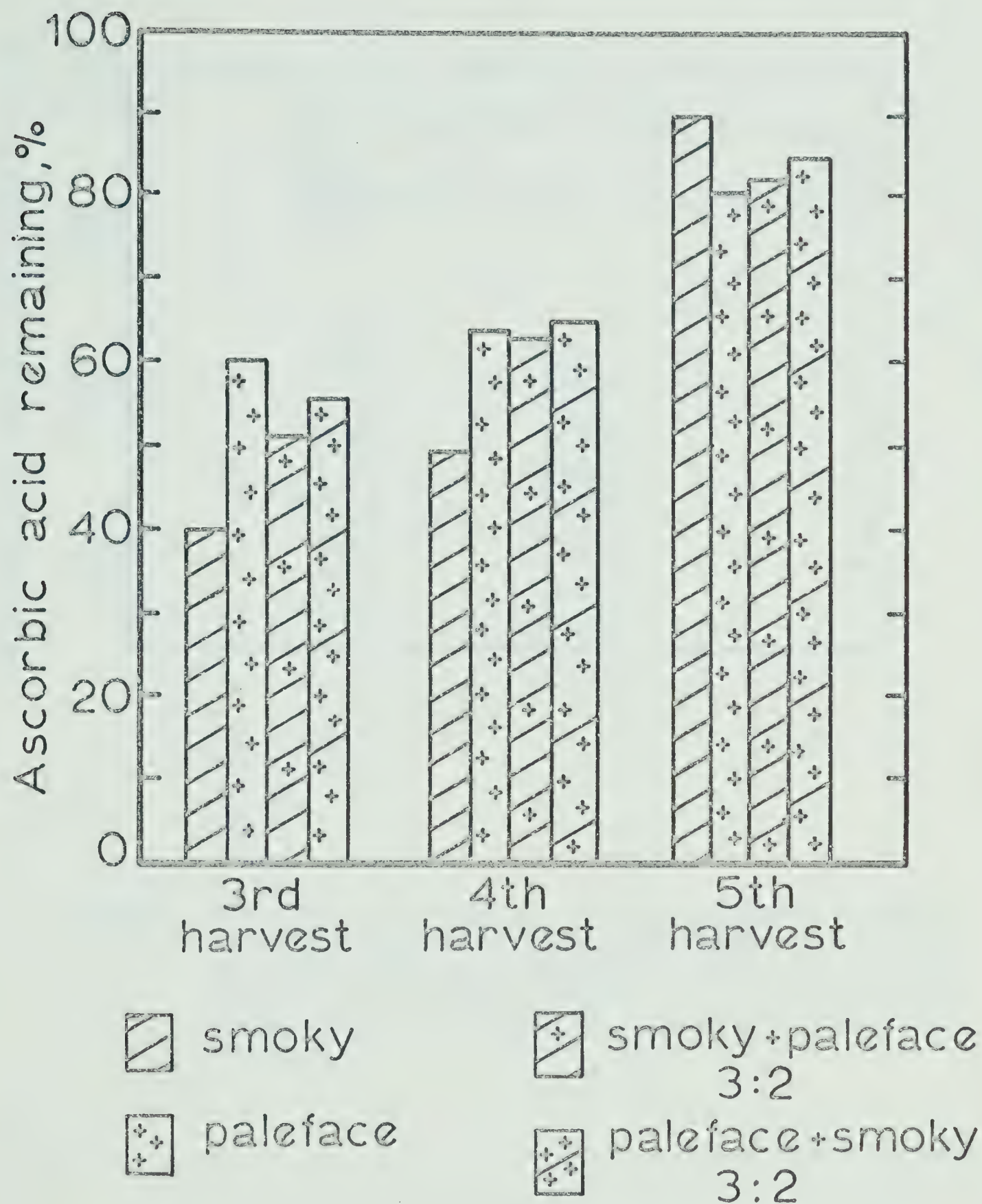


Figure 11. The effect of mixtures of berries from the Smoky and Paleface varieties on the degradation of ascorbic acid when compared to the results obtained previously for these varieties when analysed separately. Results are expressed in ascorbic acid remaining (%) after one hour of digest.

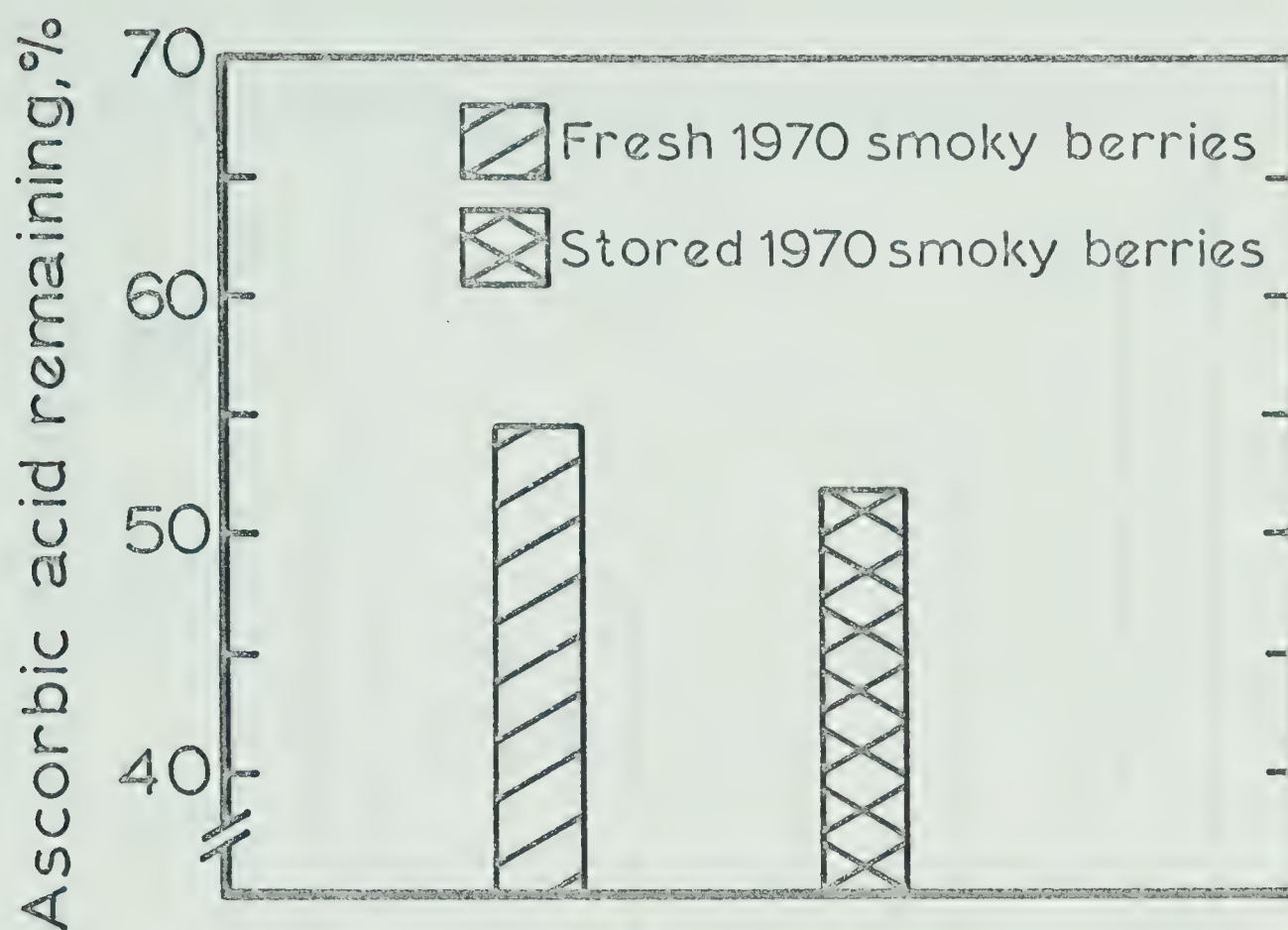


Figure 12. The effect of storage at -12°C for six months on the saskatoon berry tissue (Smoky variety) and its ability to degrade ascorbic acid, when compared to the results obtained for the fresh, unfrozen fruit. The histograms express the ascorbic acid remaining (%) after one hour of digest.

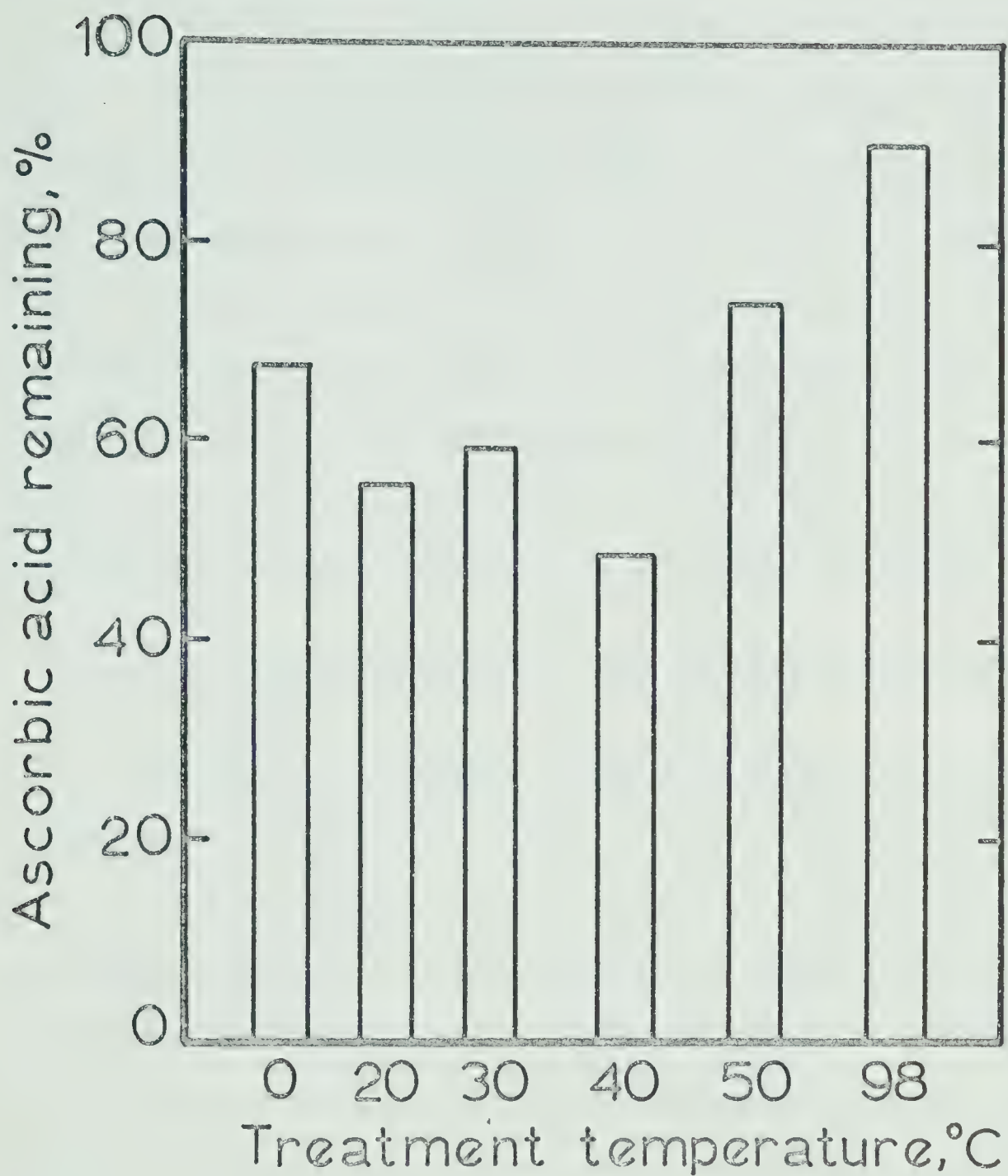


Figure 13. The effect of saskatoon extracts which had been treated at various temperatures for twenty minutes prior to their addition to the ascorbic acid solution. Graph shows the ascorbic acid remaining (%) after one hour.

gradation of ascorbic acid are shown in Figure 14. In the supernatant fractions, 80% of the ascorbic acid added initially to the saskatoon juice is still present, whereas in the fraction precipitated by the ammonium sulphate only 46% remains. The results are from two pooled experiments and represent the decay which occurs solely by the saskatoon juice. Similar assays were done to see the direct effect of ammonium sulphate on ascorbic acid itself, both the salt which formed the precipitate and also that which went into solution, that is, the supernatant fraction. The results quoted above have been corrected for this and they are expressed per 10 gm dry weight of berries.

(2) The preparation of a more sophisticated ammonium sulphate precipitate

Results of the procedure designed to evaluate the activity of an enzyme (or enzymes) which may aid the explanation of the decay of ascorbic acid are illustrated by Figure 15. The results are not expressed in the form used previously as the sample of the dialysate used, which had been obtained following the method of Dawson and Magee (1955), was equivalent to 100 gm of berries (fresh weight). From the graph, which showed ascorbic acid present versus time of analysis, slightly less than half the ascorbic acid had been degraded after one hour.

(3) Removal of microbial ascorbic acid oxidase activity

When ascorbic acid was added to the pulp of berries which had been surface sterilised with the hypochlorite for one minute, the decay observed must be due almost entirely to factors in the fruit. However, the degradation of the vitamin which was observed was almost identical

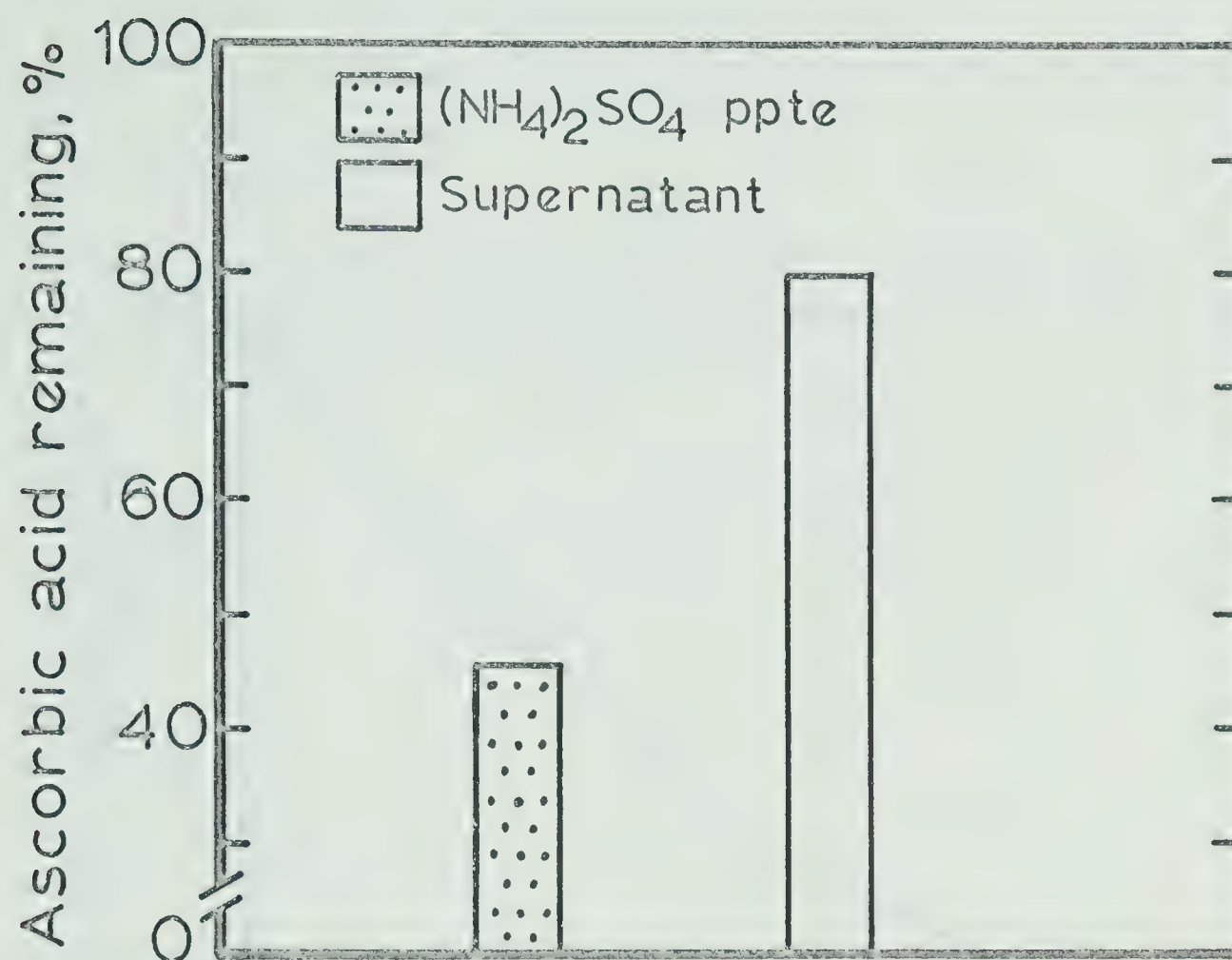


Figure 14. The effect of a crude ammonium sulphate precipitate and supernatant obtained from saskatoon juice on the degradation of ascorbic acid.

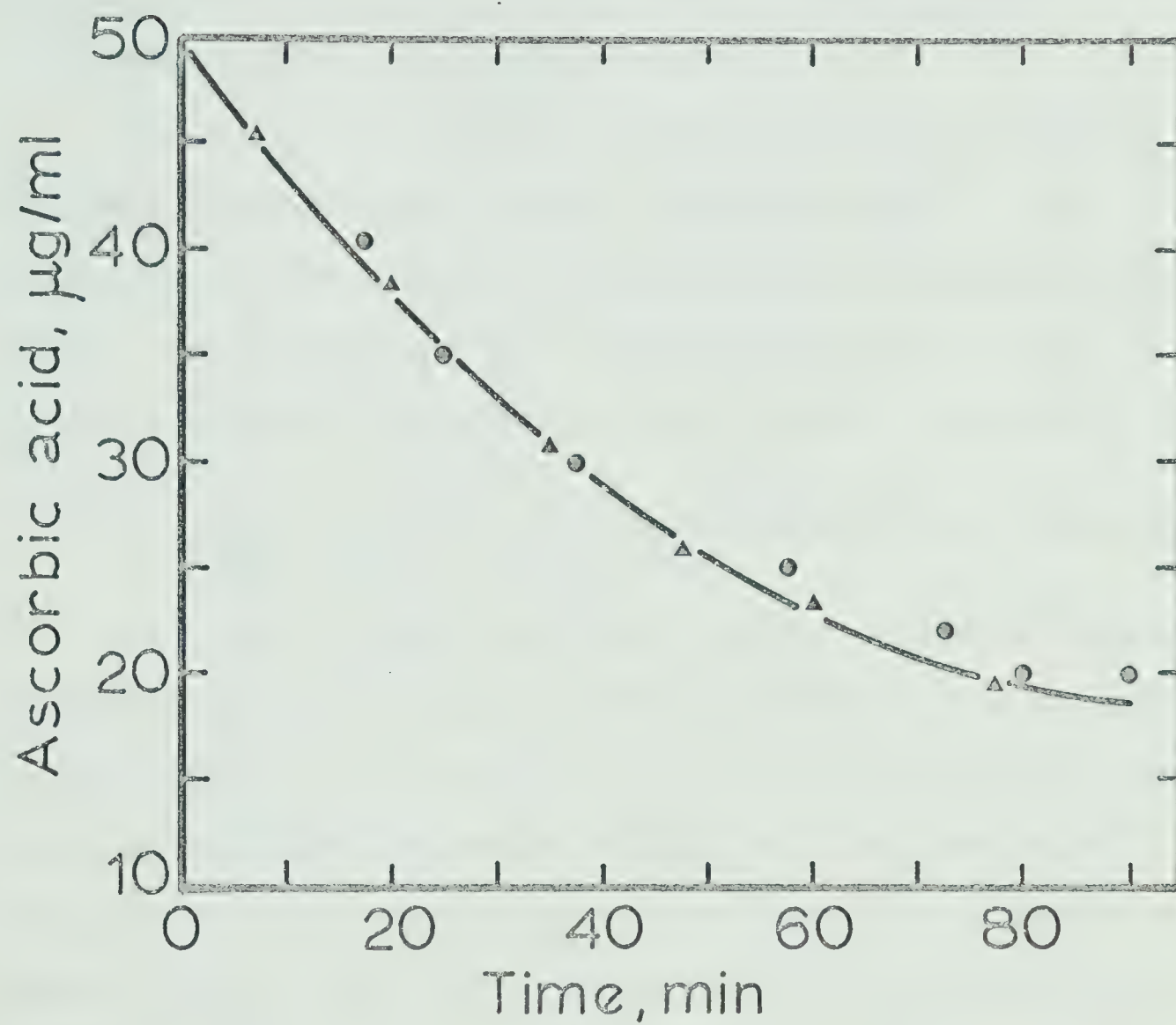


Figure 15. Graph to show the effect of a more pure ammonium sulphate precipitate on the degradation of ascorbic acid over a ninety minute period.

to that of the fresh and frozen berries. Figure 16 represents the % of ascorbic acid remaining after one hour when compared to the unsterilised fruit.

E. Copper, Phenolic Compounds and the Saskatoon Juice System

(1) The copper content of the berries

The calibration curve of the atomic absorption spectrophotometer using standard copper solutions is given in Figure 7. The graph of copper concentration in parts per million versus absorbance is linear over the range plotted. The concentration of the metal in the Smoky berries was found to be 8.9 and 9.4 ppm for two samples.

(2) The effect of copper concentrations on the decay of ascorbic acid when added to saskatoon juice

The results of these experiments have been pooled and shown on two separate Figures (Figures 17 and 18 respectively). One represents the extraction with the citrate/phosphate buffer (pH 6.6) whereas the other graph represents the results obtained using the more acidic metaphosphoric/trichloroacetic acid extraction (pH 2.4). To facilitate comparison among varieties the histograms of Paleface and Smoky have been drawn together.

The increase in copper concentration has produced a similar effect in both types of berries extracted with the citrate/phosphate buffer (pH 6.6) acid degradation. As the concentration of the metal was increased more ascorbic acid remained after one hour. When the results of the metaphosphoric acid/trichloroacetic acid buffer of pH 2.4 are considered, this reverse effect produced by the increase in copper concentration is evident for the Smoky berries, whereas for the

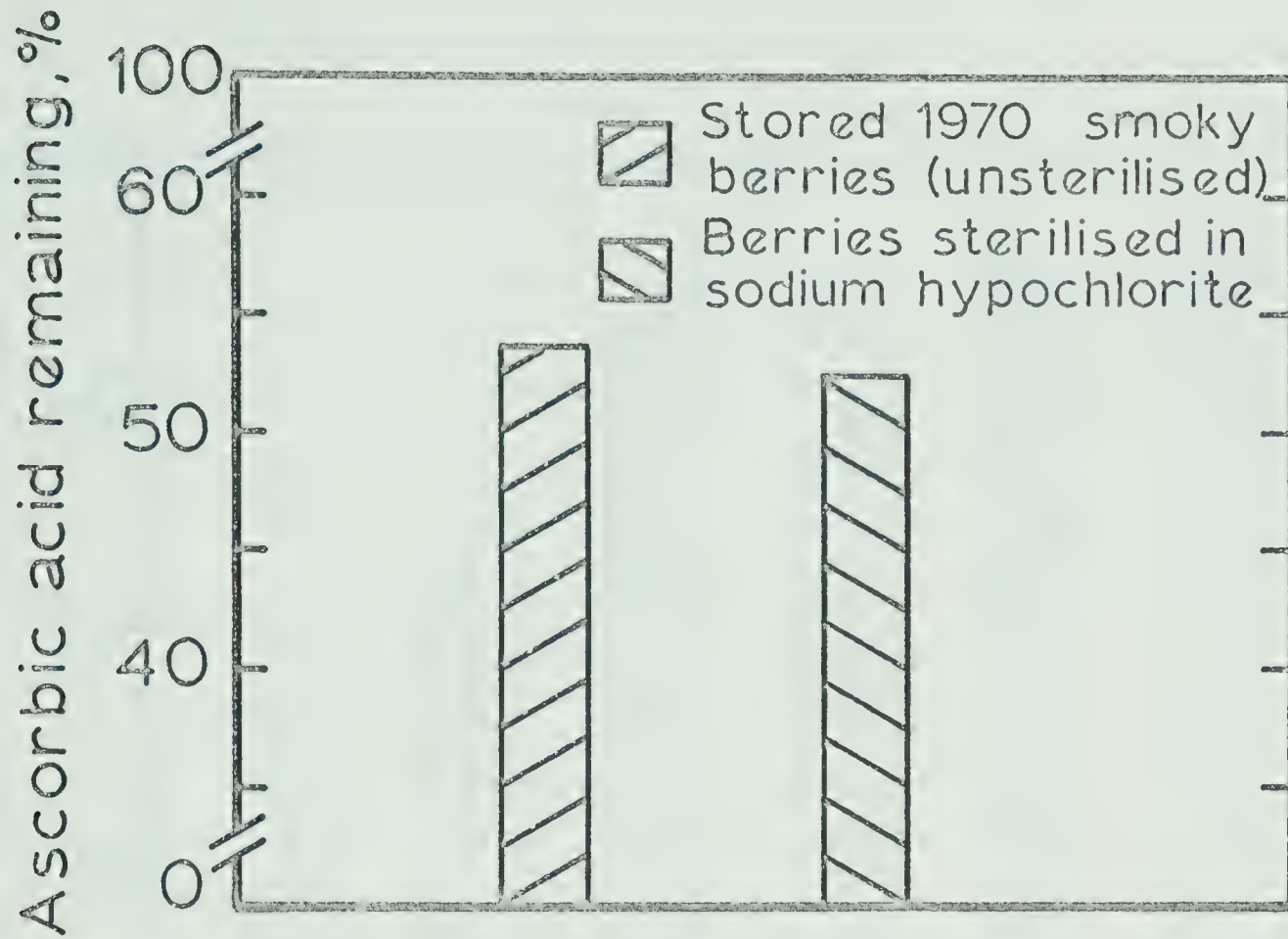


Figure 16. Ascorbic acid remaining (%) by Smoky berries which had been sterilised in sodium hypochlorite solution (5%) when compared to unsterilised fruit.

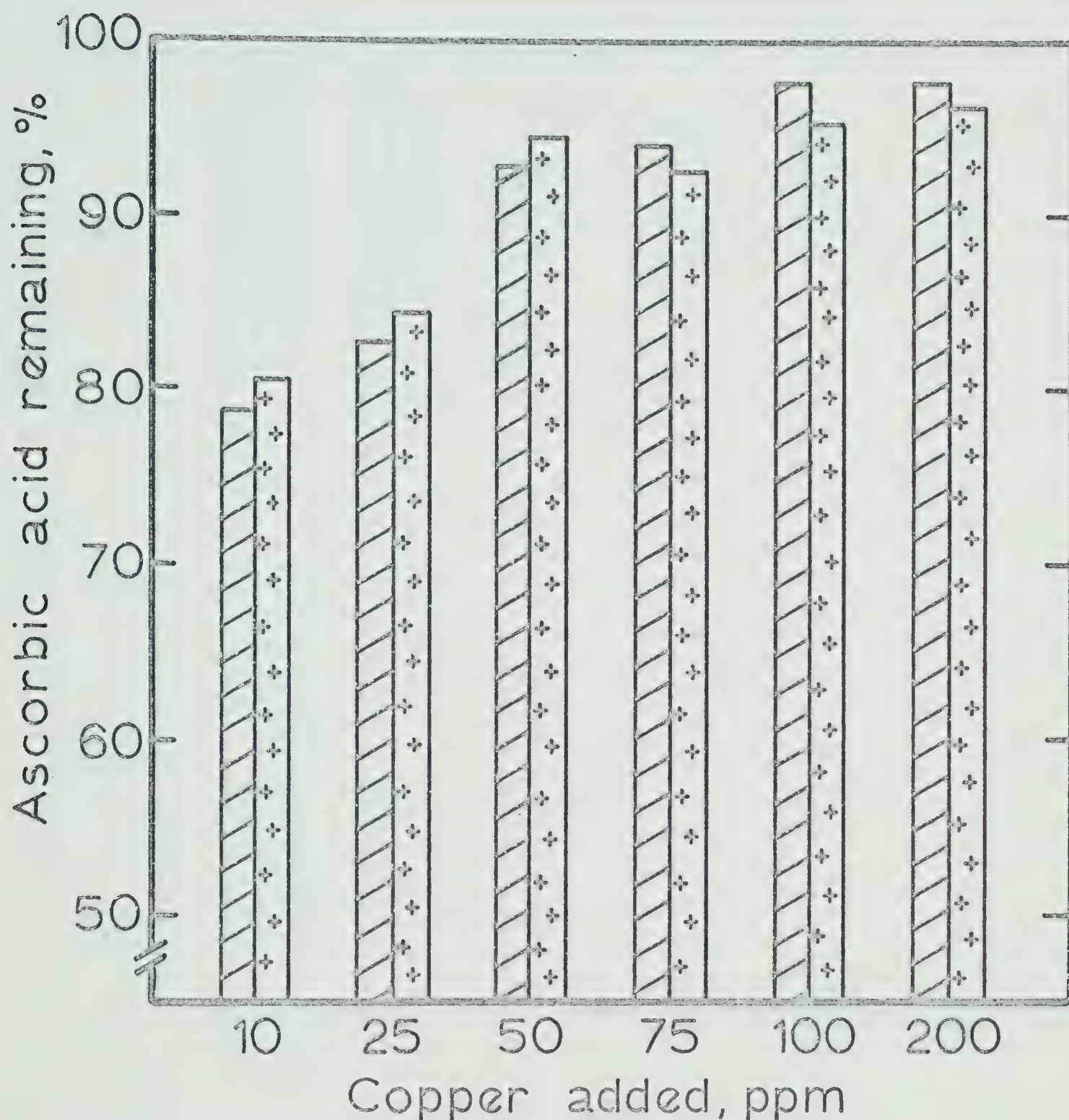


Figure 17. The effect of increasing copper concentrations on the stability of ascorbic acid when added to saskatoon berry juice from the Smoky and Paleface varieties, which had been extracted with a citrate/phosphate buffer (pH 6.6). Results are expressed as the amount of ascorbic acid remaining (%) after one hour of digest.

Key: Striped areas represent Smoky variety.
Crossed areas represent Paleface variety.

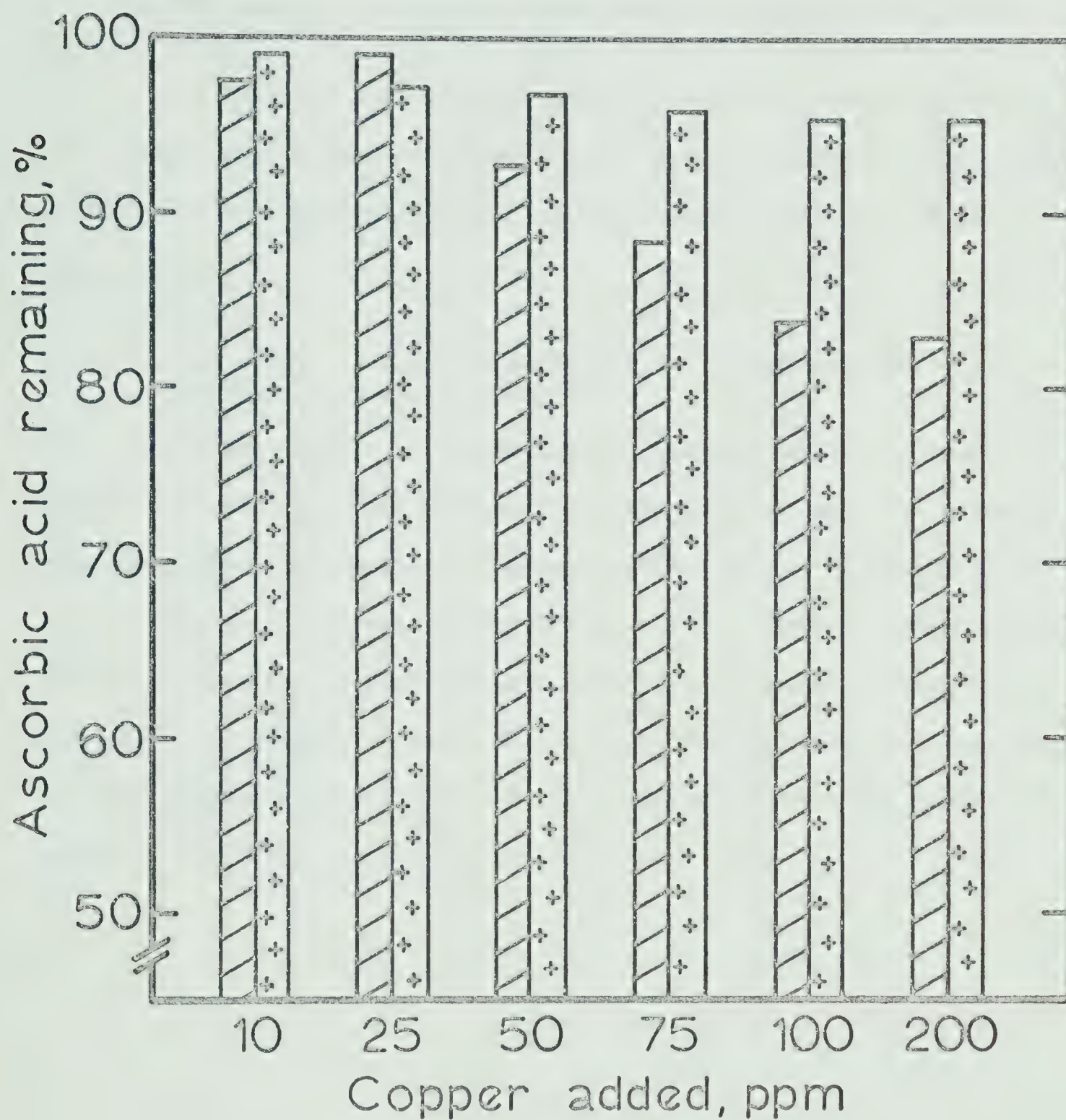


Figure 18. The effect of increasing copper concentrations on the stability of ascorbic acid when added to saskatoon berry juice from the Smoky and Paleface varieties which had been extracted with a metaphosphoric acid/trichloroacetic acid buffer (pH 2.4). Results are expressed as the amount of ascorbic acid remaining (%) after one hour of digest.

Key: Striped areas represent Smoky variety.
Crossed areas represent Paleface variety.

Paleface variety, no effect was observed.

- (3) The effect of varying concentrations of saskatoon juice on the decay of ascorbic acid in the presence of copper ions

For both the extraction procedures and also the two varieties of berries, as the concentration of juice was increased, the percentage of ascorbic acid remaining after one hour decreased (Figure 19 and Figure 20).

- (4) The effect of phenolic compounds on the degradation of ascorbic acid in saskatoon juice

The percentages of ascorbic acid remaining after one hour in extracts of the Smoky and Paleface berries, to which aglucone pigments and other phenolic components had been added are seen in Figure 21.

The general trend indicated the extracts from the Smoky berries were more affected by the compounds used than the Paleface variety. Kaempferol, a flavonol illustrated in Table 3, does provide an exception to this although the difference in the results obtained are in the order of 5%.

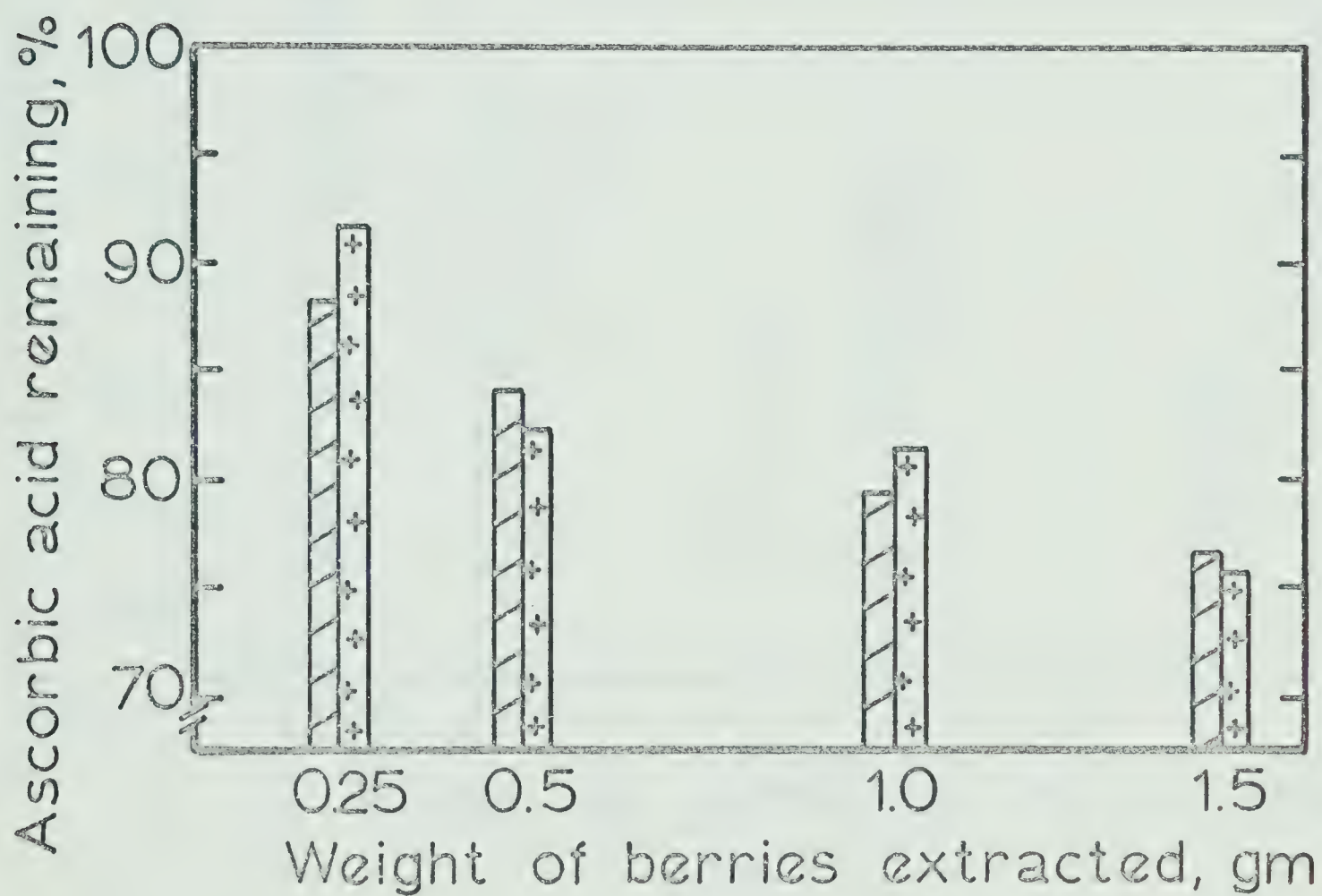


Figure 19. The effect of saskatoon juice from the Smoky and Paleface varieties which had been extracted with a citrate/phosphate buffer (pH 6.6) on the amount of ascorbic acid remaining (%) in the presence of copper ions.

Key: Striped areas represent Smoky variety.
Crossed areas represent Paleface variety.

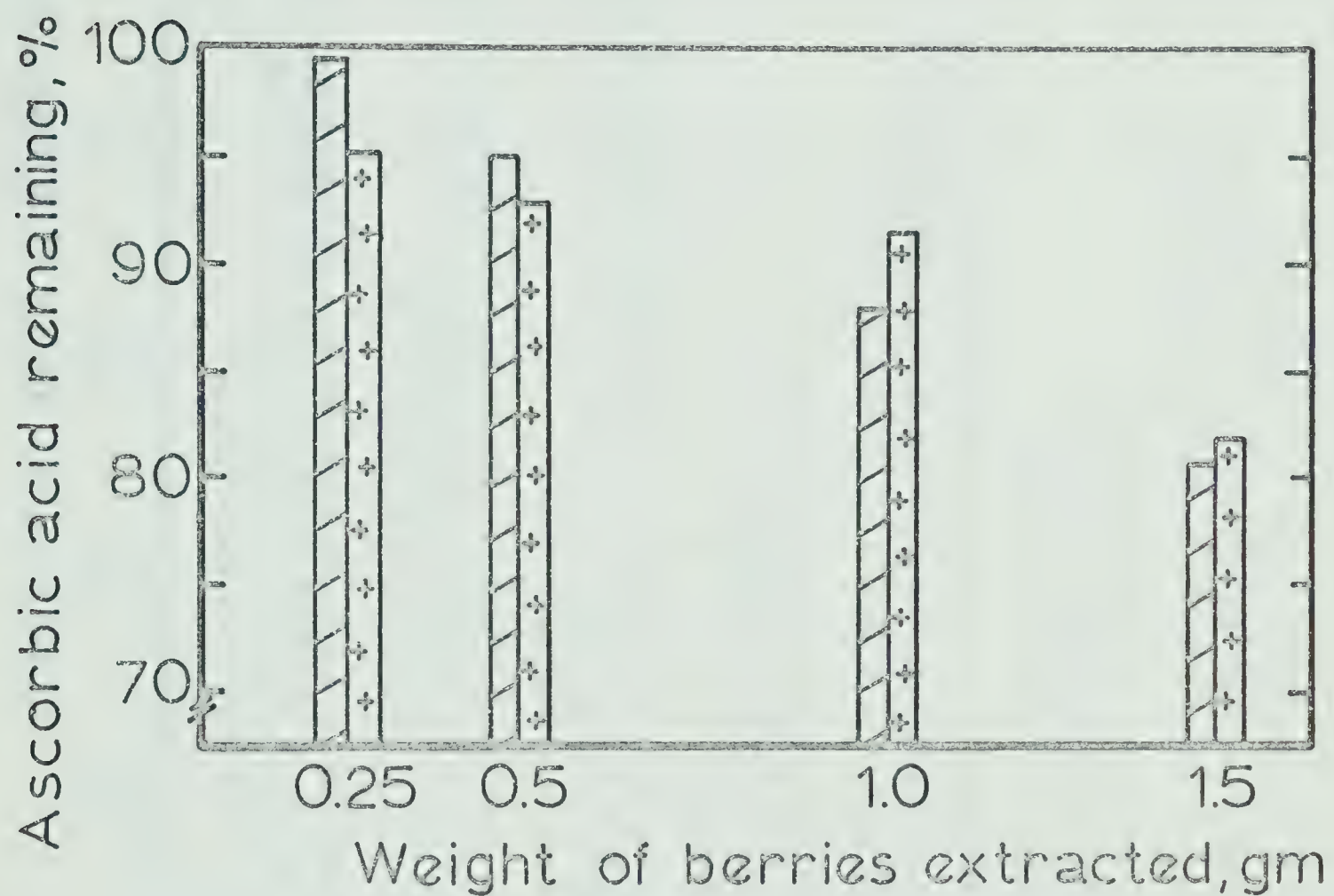


Figure 20. The effect of saskatoon juice from the Smoky and Paleface varieties which had been extracted with a metaphosphoric acid/trichloroacetic acid buffer (pH 2.4) on the amount of ascorbic acid remaining (%) in the presence of copper ions.

Key: Striped areas represent Smoky variety.
Crossed areas represent Paleface variety.

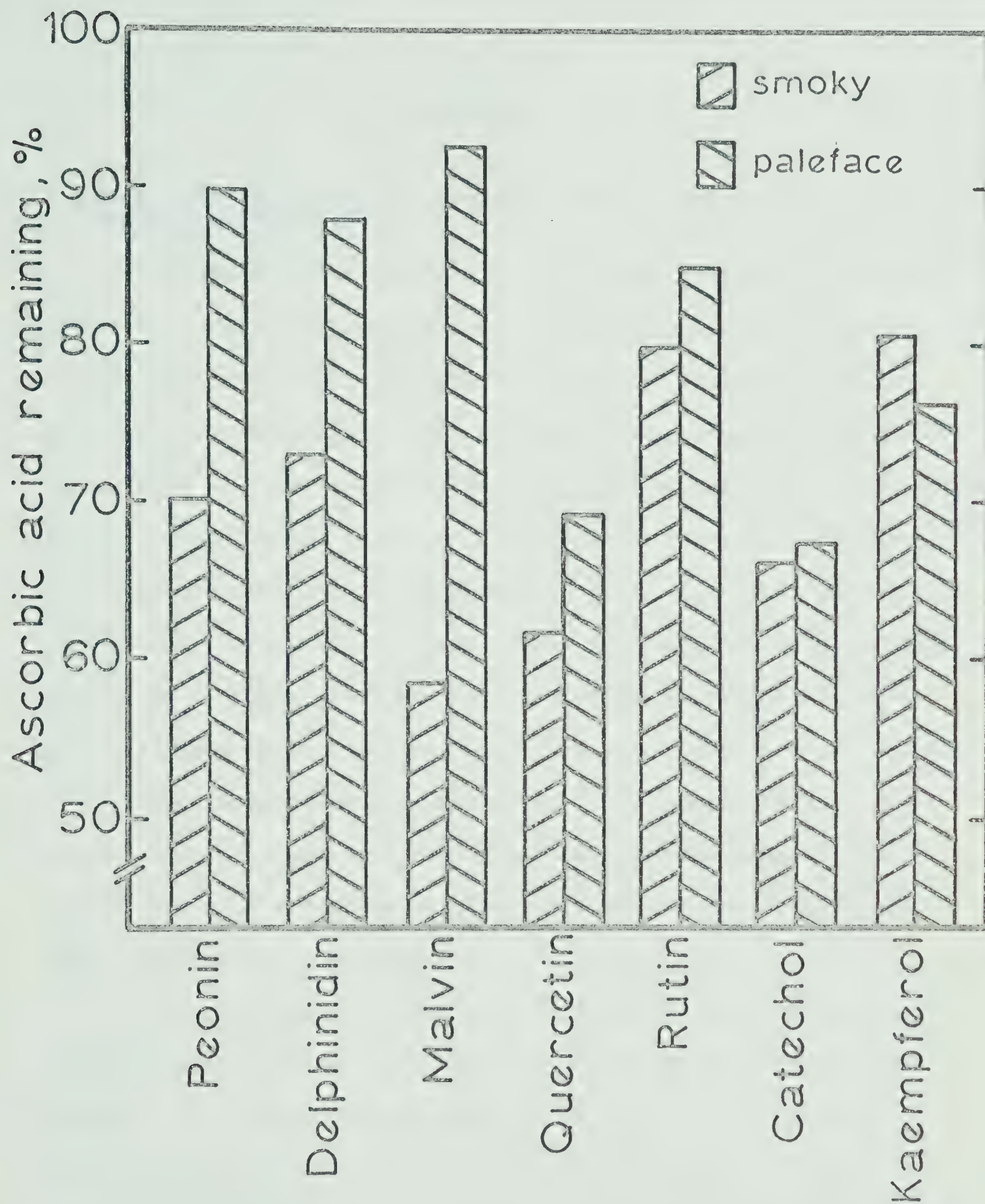


Figure 21. The effect of saskatoon juice extracts from the Smoky and Paleface varieties on the degradation of ascorbic acid in the presence of phenolic compounds. The histograms represent the amount of ascorbic acid remaining after one hour of digest for each compound.

V. DISCUSSION

A. Lack of Ascorbic Acid in the Berries and the Appearance of Dehydroascorbic Acid

The finding that ascorbic acid is absent from the berries is perhaps surprising, especially when fruits similar in appearance such as the cranberry or blueberry have been found to contain the vitamin, albeit only in small quantities. However, taxonomically, the saskatoon berry belongs to the Pomoideae, sub family of the Rosaceae, and so it is closely related to the apple, pear and quince. Hence the term "berry" is a misnomer and the fruit is in fact, a pome. Typically these fruits contain very low quantities of ascorbic acid, ranging from 0.4 - 10 mg per 100 gm of edible portion. Tuba, Hunter and Kennedy (1944) have produced contradictory results, since in a survey they detected ascorbic acid present in saskatoons in a range of 5 - 38 mg per 100 gm. The classical dichlorophenol-indophenol methods were used and in view of the difficulty in detecting any distinct end-point the validity of these results must be questioned.

Comparing the two methods for the estimation of ascorbic acid, the advantages of polarography far outweigh the classical titration methods. The decay of ascorbic acid is rapid and it is logical to assume that the more accurate results are obtained by the quicker method. Estimations were carried out in an atmosphere of nitrogen, so that the atmospheric oxidation of the vitamin is at a minimum. The unmodified method of Tillmans (1-27) was of no use because the end-point of the titration was the same colour as the smoky juice. When

Paleface berries were used in the analyses, the end-point was more accurate but it was difficult to ensure that the intensity of colour at the end-point was the same. This problem was partly overcome by the use of a spectrophotometer, but the colour of the "permanent" end-point was not stable for any of the analyses. The limits of accuracy are therefore wide and so the usefulness of the method of Robinson and Stotz is doubted.

The presence of dehydroascorbic acid was confirmed by two methods. This compound is formed from ascorbic acid by the loss of two protons (illustrated page 7). The oxidation is easily attained due presumably to a low ΔF , but if the oxidation is continued beyond the stage of dehydroascorbic acid it is irreversible as shown, so that the vitamin completely loses its potency. Ascorbic acid is not even found in the young saskatoon berries of the first harvest, so it is conceivable that immediately after the acid is formed, it is used in the plant cell and so is degraded.

The ascorbic acid content of the saskatoon berry leaves were not analysed. This species of plant could follow a pattern similar to the raspberry where the content of ascorbic acid of the leaves far exceeds that of the fruit (Fejer, Johnston, Spangelo and Hamilton, 1970). The ascorbic acid present is not translocated into the developing fruit, but it is produced and used at the shoot apex at the time of floral induction and then during the time when the fruit tissues are being differentiated (Chinoy 1967). Franke (1954) found that dried cereals and pulses contain no ascorbic acid, but once germination begins sprouting seeds form the vitamin. Following the argument of

Chinoy (1967) and Fejer et al. (1970), it seems logical that this may provide part of the solution to the absence of ascorbic acid in saskatoon berries.

The production of dehydroascorbic acid from ascorbic acid follows a two stage process, involving two separate monovalent oxidations, with monodehydroascorbic acid as an intermediate (MDHA). If the enzyme dehydroascorbic acid reductase were present in the saskatoon juice one could expect dehydroascorbic acid to be reduced by such compounds as reduced glutathione (GSH). Investigations into this compound or the activity of this enzyme may be of value. Under atmospheric conditions, Barker and Mapson (1951) found these substances to be correlated, the ascorbic acid content is dependent on the glutathione content.

B. The Decay of Ascorbic Acid when added to Saskatoon Juice and some of the Implications of this Degradation

The effect of advancing growth has proved interesting. In all varieties of saskatoon berries analysed, as maturity was approached the rate of decay of ascorbic acid by the juice has decreased. The trend is seen clearly with Smoky and the 5003 variety although it is less marked for the 0006 pink berries and also for the Paleface variety. With the four batches of Paleface berries analysed, the range of ascorbic acid remaining after one hour is small; it is of the order of 8 $\mu\text{g/ml}$. When the Smoky/5003 group of berries is considered the difference in ascorbic acid remaining from the first harvest to the last is about 30 $\mu\text{g/ml}$.

These results may be interpreted in terms of the content of plant pigments. The Smoky and 5003 berries contain the characteristic

purplish-black pigments. The 0006 variety lacks some of the darker pigments, and Paleface, as its name implies, is almost devoid of these. The pigments in the berries are largely unknown. Preliminary investigations have shown the major anthocyanins to be delphinidin ($3.0 \times 10^{-6} \text{M}$) and peonidin ($2.6 \times 10^{-6} \text{M}$) in close to equimolar concentrations (University of Alberta, 1970). Under some circumstances this group of pigments show a slight protective effect to ascorbic acid and under other conditions the effect is reversed (Clegg and Morton, 1968). However, the anthocyanins are not the only group of plant pigments which affect the oxidation of ascorbic acid (Harper, Morton and Rolfe, 1969). These authors found the flavonols affect the stability of ascorbic acid. These compounds may be present in the saskatoon berries and one might predict a wide range of similar compounds. In the case of Smoky and the 5003 berries, it seems that as the pigments are produced with advancing growth of the fruit, a protective effect is found.

Some fruits such as the potato contain colourless anthocyanins called leucoanthocyanins. It is not known if these compounds are present in the berries and what their reaction is towards ascorbic acid. The 0006 and Paleface berries may owe their lack of colour to these pigments being present instead of the more familiar anthocyanins. It does seem that the 0006 variety which contains some pigments is devoid of factors present in the smoky berries which destroy the added ascorbic acid. The 0006 and Paleface berries however, could contain a factor(s) which confer stability to ascorbic acid when it is added to the mascerated berry extract. It is known that fungal enzyme preparations from Aspergillus niger, presumably containing an anthocyanase

enzyme, exert a significant effect on decolourising the anthocyanins in berries (Huang 1955). This process involves the hydrolysis of the anthocyanin into sugar and aglucone, and a spontaneous transformation of the latter into colourless derivatives. This could provide an explanation for the results observed above which may involve these derivatives, although the microbiological study of the surface of the berries showed little bacterial or fungal contamination so the probability of an anthocyanase is remote. However, the enzyme is also found in plants, so the possibility of this enzyme being present in the saskatoon berries does exist.

When mixtures of berries were analysed together, the effect on added ascorbic acid was similar for each harvest of fruit. A general trend was seen, in that slight protection of ascorbic acid was given by the Paleface berries. The vitamin may be prevented from undergoing oxidation by factors present in the juice, or these colourless berries merely diluted the effect produced by the purple Smoky variety. For each harvest this trend was continued and magnified.

Freezing the berries, storing them and later the effect of thawing did not produce any observable changes in the reaction of the fruit juice to the added ascorbic acid solution. It may be concluded that the plant pigments are unaffected by extremely low temperatures, or if an enzyme is responsible for the degradation process, its catalytic ability is not impaired.

The amount of ascorbic acid degraded by juice treated beforehand at various temperatures decreased with increasing temperature. The assumption that one of the factors contributing to the decay of

ascorbic acid is proteinaceous has been partially substantiated in that the effect may be interpreted to be due to thermal denaturation of an enzyme protein. Little degradation of the initial concentration of ascorbic acid occurred with one sample which had been boiled, whereas under the same conditions at 40°C, only 50% of the original ascorbate solution remained. At 0°C slightly more ascorbic acid remained. The optimum temperature of activity for the unknown factors in the crude juice extract lies somewhere between 30 - 40°C, since at 50°C the effect of thermal inactivation is apparent. The plant pigments alone are stable to heat and processing temperatures, although the effect of other plant materials such as pectins, polysaccharides, proteins etc. remains obscure. One of the most obvious explanations for this effect is the change which is induced in a protein fraction in the berries.

Many plants have been found to contain an ascorbic acid oxidase which catalyses the oxidation of ascorbic acid. This system could exist in the saskatoon berries. It is known that dehydroascorbic acid can be reduced back to ascorbic acid by agents such as sulphides and also with the help of a reductase. If it can be supposed that the reaction occurs by a reductase in the berries, substrate is provided for the ascorbate oxidase to act upon immediately. However, since the actual situation in vivo is unknown, one assumes that both enzymes are located in similar sites in the plant tissue. The location of the reductase is unknown, but Hallaway, Phethean and Taggart (1970) found ascorbate oxidase in both the cell wall and also the soluble fraction of cabbage leaf tissue.

The results expressed in Figures 4 and 5 indicates that the saskatoon berry is not peculiar in this facet--the fruit tissue contains an

ascorbate oxidase enzyme. By the addition of ammonium sulphate to the point of saturation, the proteins were salted out and separated by centrifugation. It is expected that the larger protein molecules present in the juice are carried down into the salt pellet and the centrifuge speed used to obtain this was not great enough to precipitate the mitochondrial or the microsomal fractions although speeds generally used to obtain these two fractions are of the order of 10,000 x g and 100,000 x g respectively. For the preliminary experiment slightly higher speeds were used and perhaps more mitochondria were precipitated.

When the combined supernatants and the combined pellets of the initial experiment were added to solutions containing ascorbic acid, decay was observed in both cases which was greater than simple autoxidation of ascorbic acid alone. Since the greater rate of decay was observed by the ammonium sulphate pellet, it would seem that the protein fraction is the causative factor.

It is known that plant pigments affect the stability of ascorbic acid (Clegg and Morton, 1968; Harper 1969; Harper, Morton and Rolfe, 1969). Investigations into the decay of ascorbic acid caused by proteins in the juice system is complicated by the fact that the crude ammonium sulphate pellet used contained some of the pigments to which the saskatoons owed their colour.

The method of Dawson and Magee (1955) produced similar decay of ascorbic acid. In this procedure most of visible pigments had been lost after dialysis for thirty-six hours although some pigments remained.

The plant pigments are generally of small molecular weight. Delphinidin appears to be one of the principal anthocyanins in saskatoon

berries, and has a molecular weight of about 339. Compounds in this weight range were expected to be lost after the dialysis stage, however they are frequently attached to glucoside residues and it would seem that some are firmly, if not permanently, attached to protein moieties or other molecules precipitated by the ammonium sulphate. Therefore it has been impossible to separate the pigments from the protein fraction. To try to solve this problem the method of ascorbic acid oxidase assay of Porath et al. (1967) was used. This involved the use of Sephadex G-100 and G-200 prepared in pyrex columns 60 cm x 2.8 cm, but no separation was achieved as the pigments gradually bled throughout the column. When Sephadex G-10 and G-25 were used, a tailing effect was still obtained which would seem to enhance the idea that the plant pigments are tightly bound to protein moieties and other molecules of high molecular weight.

There seems little possibility that the decay of ascorbic acid which has been noticed can be due to a microbial source. Literature has been reviewed in this connection (White and Smith, 1962; White and Krupka, 1965; Volk and Larsen, 1963). The berries were handpicked and packaged, so the possibility of contamination by coliform organisms cannot be overlooked. Despite this, aerial contamination of bacteria and fungi, etc. by wind and dust could have occurred. But from Figure 9 and Figure 10 where the decay of ascorbic acid added to batches of berries from the various harvests are compared, the first batch of berries harvested seemed to produce the greatest degradation of the acid. At this stage of maturity when the berries had not been formed for many days, microbial contamination is expected to be at a minimum.

Whereas in the fourth and fifth batches of berries which were exposed to the environment for a much longer time, one might expect a higher degree of contamination.

Treatment for one minute in sodium hypochlorite solution was found to be an effective surface sterilising procedure for the saskatoon berries. It is feasible that after the disruption of the bacterial and fungal cell walls, enzymes may still be in operation, but it is unlikely that enzymes from about 600 organisms are likely to produce any effect.

Under the conditions of the experiment where juice was added to the ascorbate solution, the complete experiment lasted for approximately 100 minutes, and the multiplication of microbes would not be expected to produce a noticeable effect. This reasoning was confirmed by surface sterilisation with the sodium hypochlorite as the percentage of ascorbic acid remaining after one hour was identical to that observed previously.

C. Copper, Phenolic Compounds and the Saskatoon Juice System

The role of copper in the systems which are known to exist for the decay of ascorbic acid are diverse, both in the role of an ascorbic acid oxidase and in the non-enzymatic reactions which can occur. The copper concentration of two samples of Smoky berries (found to be 8.9 and 9.4 ppm) may reflect to some extent the growing conditions. Exact data for the content of copper in the soil where the saskatoons were grown is not available.

When the concentration of copper is increased beyond physiological levels, the effect produced varies depending on the

extraction medium. In the metaphosphoric acid/trichloroacetic acid buffer (pH 2.4) the ascorbate oxidase is assumed to be more or less inactive since the metaphosphoric acid forms a non-ionisable copper complex, and so the factors causing the non-enzymatic decay are predominating. The citrate-phosphate buffer (pH 6.6) was the medium used by Porath, Samorodova-Bianki and Hjerten (1967) to extract and preserve ascorbic acid oxidase from Cucurbita pepo. According to Harper, Morton and Rolfe (1969) at this pH range the reaction between ascorbate, copper and plant pigments is minimal, so in the main, the changes which are taking place are due to an enzyme. However, the possibility that non-enzymic changes could take place cannot be totally excluded.

Extraction with the more acidic buffer has produced quite different effects for the Smoky and Paleface berries (Figure 18). The Paleface berries were quite unaffected as the concentration of copper was increased. Under these conditions the decay of ascorbic acid is increased by the increasing copper content in Smoky berries. One feasible solution to this could be found in a more detailed study of the pigments present in both berries.

In the Smoky berries the levels of copper used need not be sufficient to hasten the non-enzymic reaction alone to completion. The decay which is observed may be the reaction which occurs in the fruit as a result of the accelerated oxidation by the visible purple pigments. However, Clegg and Morton (1968) have found that in the presence of copper concentrations of this order, the oxidation of ascorbic acid is not attributed to anthocyanins but to flavonols and other plant pigments.

These investigations also indicated that under such conditions, the anthocyanins as glucoside derivatives showed a slight protective effect.

The effect of copper on the citrate/phosphate buffer extract is quite the reverse. For both varieties of berry, as copper is added, the degradation of ascorbic acid is reduced. Ascorbic acid oxidase, as mentioned, has a copper containing prosthetic group. The copper content is 0.25%. During extraction the protein moiety may unfold, and copper ions might be lost as the bond between the copper and the enzyme protein may be ruptured. However, at pH 6.6, in a citrate-phosphate buffer oxidase activity has been found. Loosening of the enzyme structure may occur which could then account for the exchange and uptake of copper ions, which must take place for the consolidation of the enzyme structure to form a fully functioning holoenzyme.

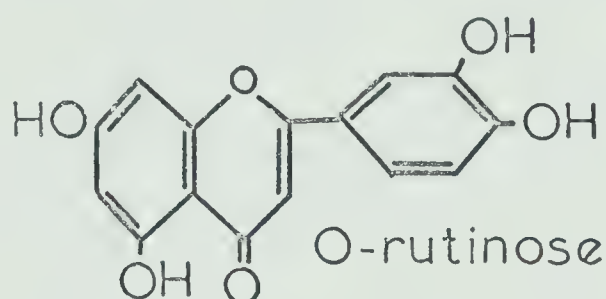
The effect of increasing the content of juice from both extractions to the standard ascorbate-copper solutions is as expected. As the concentration of juice is increased, there is a concomitant increase in the factor or factors which are responsible for the decay of ascorbic acid (Figure 19 and Figure 20). The structure of the anthocyanidins, flavonols, and flavan-3-ols which were added to the metaphosphoric acid/trichloroacetic acid berry extract, (pH 2.4), are given in Table 3. These compounds were added at concentrations of 1.0×10^{-4} M. This figure was arbitrarily chosen, but it did represent one of the main concentrations used in a series of similar experiments by Morton (1968), Harper (1969) and Harper, Morton and Rolfe (1969).

Several conclusions can be tentatively drawn from Figure 21. When the results are compared to Figure 9 and Figure 10, for the berries

harvested on July 30, 1970, it can be seen that in all cases the concentration of phenolic compounds added showed a sparing effect on ascorbic acid. The ascorbic acid which remained after one hour is greater when pigments were added to the mulch. However, as before, the differences between the two varieties of berries still existed. The three anthocyanidins, peonin, delphinidin and malvin provided the greatest stability to ascorbic acid in the Paleface berries. The difference of 20% or more ascorbic acid remaining after one hour observed between the Smoky and Paleface berries could reflect an optimum concentration of total anthocyanidins has been achieved in terms of ascorbic acid protection. The degradation of the compound observed in the Paleface berries was little more than the autoxidation of ascorbic acid itself. For the Smoky berries the concentration of 1.0×10^{-4} M did not achieve this result.

These results do in part confirm the experiments of Clegg and Morton (1968) cited earlier. These investigators studied the effect of anthocyanin pigments that is, a sugar moiety attached to the position 3 of the benzopyrilium nucleus of the anthocyanidin molecule, both in the presence and absence of copper ions. Clegg and Morton (1968) observed that without the addition of copper ions, the anthocyanins accelerated the oxidation of ascorbic acid. It could be assumed that the concentration of copper of about 9.0 ppm found in the berries hastened the oxidation of ascorbic acid under the conditions used initially (Figure 8) and once the anthocyanins were added any free radical formation which formerly brought about the degradation of ascorbic acid was now at a minimum.

The key to the problem may indeed be linked with the presence and/or the nature of the sugar residue attached to the aglucone. Reference is made to the flavonoids rutin and quercetin which were used in the study, as rutin is composed of the aglucone, quercetin, and rhamnose and glucose, a pentose and hexose respectively. These two sugars bound together are called rutinose and then attached to the aglucone in position 3 as seen below:

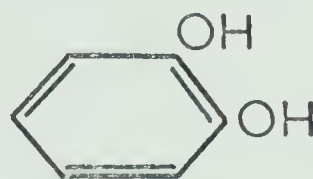


Quercetin and rutin, like the anthocyanidins could also act as good chelating agents for copper. The effect of rutin and quercetin on the amount of ascorbic acid remaining in the saskatoon juice system are quite different to the anthocyanins. For both varieties of berries following rutin addition 20% more ascorbic acid was detected by the polarographic trace after one hour than with quercetin. It would appear therefore that although these two flavonols are similar in structure the presence of sugar residues is of importance in the system with regard to the stability of ascorbic acid. Work in this context would be valuable to identify the sugar residue(s), if any, which are attached to the varying plant pigments. The sugar moieties may be different in each case and perhaps linked in different sequences as di- or trisaccharides.

The effect of catechol, a simple phenol with structure seen below, produced more or less identical results for both varieties of

berries--where about 68% of the ascorbic acid added initially remained. It would seem that the concentration of this phenol added was not optimum for protection of ascorbic acid. The possibility could exist where the concentration added was in excess of the amount required for heavy metal chelation and the net result was a decrease in the percentage of ascorbic acid remaining.

Catechol:



Catechol is widely distributed in fruits and vegetables and it has been known for some years that this and similar compounds may be formed by the breakdown of anthocyanins (Onslow 1920). Under the conditions of the experiment the catechol could have been converted into further compounds which could themselves exert some effect on the stability of ascorbic acid. The enzyme catecholase which is a polyphenol-oxidase is just one of the enzymes widespread in plants which catalyses the oxidation of catechol into other similar phenolic compounds. These enzymes are generally similar to ascorbic acid oxidase, since they possess copper as part of their prosthetic group.

Chelation of heavy metals such as copper is generally associated with the presence of hydroxyl groups on the 'B' ring of the flavonoid structure at positions 3'4' (Table 3) Clemetson and Anderson (1966). Kaempferol and quercetin are both members of the flavonol group of compounds with hydroxyl groups on the 4' (kaempferol) and 3' and 4' (quercetin) positions of the 'B' ring. From the work of Clemetson and Anderson (1966) one would assume the oxidation of ascorbic acid in the presence of copper and quercetin to be minimal. However, the percentage

of ascorbic acid remaining after one hour in the saskatoon extracts to which kaempferol was added was greater than the result obtained using quercetin. Other mechanisms must be in operation in the saskatoon juice system. The actual interaction of the flavonol compounds with other flavonoids and plant components is not known.

VI. SUMMARY

A. The usefulness of the colourimetric technique for ascorbic acid assay in saskatoon berries, using 2,6-dichlorophenolindophenol was questioned, because of an inability to detect an end point in titration. The polarographic method of ascorbic acid estimation was preferred using an acetate electrolyte at pH 5.5.

B. Ascorbic acid was not detected in saskatoon berries but dehydroascorbic acid was detected. Two reduction methods involving the use of 2,3-dimercaptopropanol and α, α' -dipyridyl confirmed this compound was present in the fruit in concentrations of the order of 22-24 mg per 100 gm (fresh weight) respectively.

C. When synthetic ascorbic acid was added to the juice from saskatoon berries, polarographic assays showed the vitamin was degraded rapidly. The extent of the destruction varied with the variety of berry and time of harvest, being greatest in the immature berries. This phenomenon was not affected by freezing the fruit at -12°C , however when ascorbic acid was added to berry extracts which had been boiled for twenty minutes, degradation was minimal.

D. An ascorbic acid oxidase enzyme which accounted for part of the degradation of ascorbic acid was found to be inherent in the Smoky variety. The use of a Sephadex gel filtration procedure for the extraction of the enzyme molecules on the basis of molecular weight proved fruitless. Several sizes of Sephadex beads failed to separate the small plant pigment molecules from the protein fraction.

E. The copper content of the berries was of the order of 9 ppm. By increasing this concentration beyond physiological levels, the degradation of ascorbic acid by juice extracts was affected. The effect observed depended on the pH of the extraction medium. At pH 6.6 when the nonenzymic degradation is presumed minimal for both Smoky and Paleface berries, as the copper concentration was increased the degradation of the vitamin increased concomitantly. Whereas, at pH 2.4 enzymic degradation is minimal, and when the copper concentration was increased protection for ascorbic acid was observed in the Smoky berries although the Paleface berries were unaffected.

F. Phenolic compounds showed a sparing effect on the degradation of synthetic ascorbic acid by mature Smoky and Paleface saskatoons. The anthocyanidins peonin, dephinidin and malvin provided the greatest stability to ascorbic acid in the Paleface berries. Twenty percent more ascorbic acid was detected by the polarographic trace following rutin addition than when its aglucone component, quercetin was used. The effect of catechol addition was similar for both varieties.

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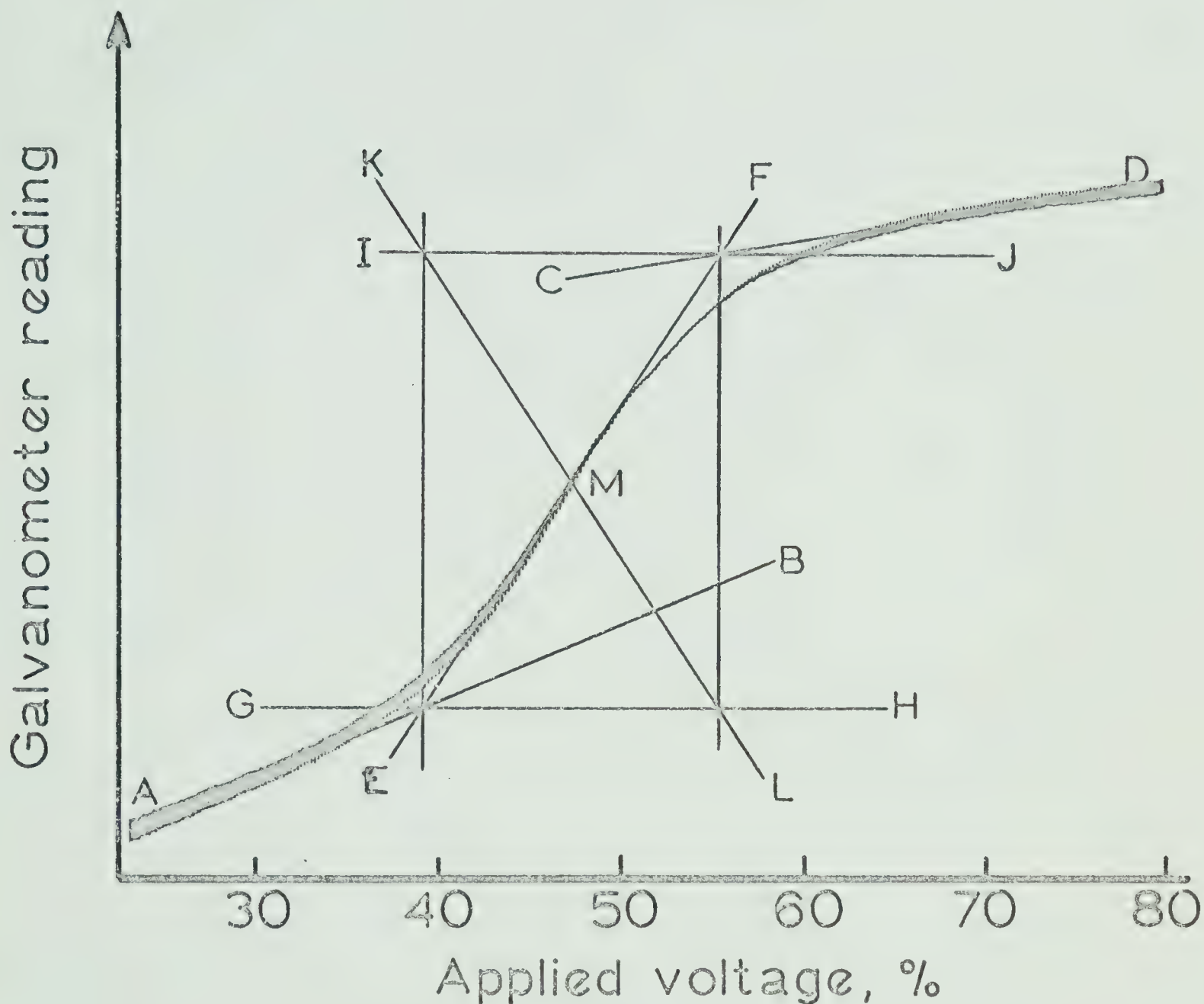
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Appendix I. The extrapolation method to evaluate a polarographic trace quantitatively.

Conditions: Electrolyte used: Sodium acetate (2N) acetic acid (2N)
of pH 5.5

Current sensitivity: 0.015

Voltage range: -5 ----> +5

Voltage applied (%): 20 -----> 80

Procedure to determine the height of the polarographic wave: line A-B represents the residual current i.e. the electrolyte line C-D represents the total or limiting current plateau. Measurement of the diffusion current follows in order of the alphabetical construction of the lines as shown. The distance (cm) between the lines I-J and G-H is an index of ascorbic acid concentration and from this the half wave potential, M, can be estimated.

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